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(54) **ANTICOAGULANT POLYPEPTIDES.**

(57) Recombinant human urinary thrombomodulin and variant polypeptides produced as a result of replacement, deficiency, addition and like operations of amino acids in part of the amino acid sequence of the thrombomodulin, having a capability of binding with thrombin and anticoagulant and thrombolytic activities. They can be produced efficiently in large amounts by the gene recombination techniques, and are useful for preventing and treating diseases which participate in an increase of blood coagulation, because they are free from adverse effects such as induction of hemorrhage.

EP 0 489 180 A1

Field of the Invention

This invention relates to a novel polypeptide obtained by genetic recombinantion techniques, having activities similar to human thrombomodulin such as anticoagulant activity and thrombolytic activity, to a deoxyribonucleic acid (to be referred to as "DNA" hereinafter) fragment which encodes said polypeptide and to a process for the production of said polypeptide by means of genetic recombinantion techniques. This invention also relates to an agent for use in the prevention and/or treatment of hypercoagulability related diseases which comprises said polypeptide as an active ingredient.

Background of the Invention

Heparin, antithrombin III and the like are currently used as anticoagulants. With regard to thrombolytic agents, urokinase isolated from urine or cultured kidney cells, streptokinase isolated from β-hemolytic streptococcus and the like have been put into practical use, as well as a recently developed tissue plasminogen activator.

These substances, however, have side effects such as bleeding tendency and show only one activity, anticoagulant activity or thrombolytic activity.

Recently, in the field of fundamental studies, a substance having an effect to inhibit blood coagulation and an effect to enhance formation of activated protein C which enhances fibrinolysis has been found in a rabbit lung tissue extract by N. L. Esmon *et al.* and named thrombomodulin (*J. Biol. Chem.*, Vol.257, p.859, 1982). It has been reported by Maruyama *et al.* that thrombomodulin is a thrombin receptor localized on blood vessel endothelial cells and that thrombin is deprived of its blood coagulation activity when bonded to thrombomodulin and the thrombin- thrombomodulin complex activates protein C to impart its anticoagulation effect (*J. Clin. Invest.*, Vol.75, p.987, 1985). In other words, it is possible that thrombomodulin imparts effects of both inhibiting blood coagulation and enhancing fibrinolysis and therefore can be applied to clinical means.

The following summarizes examples on the isolation of human thrombomodulin so far reported. In this instance, unless otherwise noted, data on the molecular weight cited below are those measured under non-reducing conditions by means of sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE).

P. W. Majerus *et al.* have purified thrombomodulin from human placenta and reported its a molecular weight as 75 K (*J. Biol. Chem.*, Vol.259, p.12246, 1984), while Aoki *et al.* have purified thrombomodulin from human placenta and reported its molecular weight as 71 K (*Thrombosis Res.*, Vol.37, p.353, 1985; and Japanese Patent Application Kokai No. 60-199819). Maruyama *et al.* have purified thrombomodulin from human lung and reported that its properties were the same as those of placenta origin (*J. Clin. Invest.*, Vol.75, p.987, 1985). In addition, Suzuki *et al.* have partially purified thrombomodulin from human platelet and determined its molecular weight as 78 K and reported that the thrombomodulin preparations obtained from platelet, placenta and lung blood vessel endothelial cells had the same properties in terms of their electrophoretic behavior and affinities for thrombin and protein C (*J. Biochem.*, Vol.104, p.628, 1988).

In addition to these thrombomodulin molecules purified from human organs (to be referred to as "human thrombomodulin" hereinafter), the following substances having similar properties (to be referred to as "human thrombomodulin- like substance" hereinafter) have been reported.

P. W. Majerus *et al.* have partially purified two human thrombomodulin- like substances from a human plasma having molecular weights of 63 K and 54 K, respectively, and reported that similar substances existed also in urine (*J. Clin. Invest.*, Vol.75, p.2178, 1985). In addition, Ishii *et al.* have reported that similar substances having molecular weights of 105 K, 63 K, 60 K, 33 K, 31 K and 28 K (no description about reducing or non- reducing measuring condition) were excreted into urine (Abstracts of Papers, the 108th Meeting of Pharmaceutical Society of Japan, 6F05, 11-1, 1988). Other examples of human thrombomodulin-like substances obtained from urine include a mixture of substances having molecular weight of 200 K, 48 K and 40 K (Japanese Patent Application Kokai No. 63-30423) and those having molecular weight of 39 K and 31 K (Japanese Patent Application Kokai No. 63-146898).

C. T. Esmon *et al.* have prepared a chemically synthesized peptide which corresponds to a portion of the thrombomodulin molecule (Japanese Patent Application Kokai No. 2-19399).

On the other hand, Suzuki *et al.* have cloned a gene of human thrombomodulin precursor containing a signal peptide from a human lung cDNA library making use of genetic engineering techniques, determined entire structure of the gene and revealed an amino acid sequence of 557 amino acid residues with a signal peptide of 18 amino acids adjacent to the sequence, with a conclusion that the N terminal amino acid sequence of human thrombomodulin was Ala Pro Ala Glu Pro (*EMBO Journal*, Vol.6, p.1891, 1987). In addition, Suzuki *et al.* have reported that activity of the human thrombomodulin prepared by genetic

engineering techniques was the same as that of natural human thrombomodulin purified from biological tissues (*J. Biol. Chem.,* Vol.264, p.4872, 1989) and that the human thrombomodulin- like activity was restricted to a portion of the amino acid sequence, from 345 position to 462 position amino acid residues numbered from its amino terminus, and the activity disappeared when any amino acid in the active portion was deleted (*J. Biol. Chem.,* Vol.264, p.10351, 1989; and Abstracts of Papers, the 12th Meeting of International Society of Thrombosis and Hemostasis, p.334, Title No.1039, 1989). Also, R. W. Jackman *et al.* have determined complete structure of a gene of human thrombomodulin precursor and revealed an amino acid sequence of 559 amino acid residues with a signal peptide of 16 amino acids adjacent to the sequence, with a conclusion that the N terminal amino acid sequence of human thrombomodulin was Phe Pro Ala Pro Ala Glu Pro (*Proc. Natl. Acad. Sci.* USA, Vol.84, p.6425, 1987). Also, D. Wen *et al.* have cloned a gene of thrombomodulin precursor from a human umbilical cord vein cDNA library, determined complete structure of the gene and revealed an amino acid sequence of 554 amino acid residues with a signal peptide of 21 amino acids adjacent to the sequence, with a conclusion that the N terminal amino acid sequence of human thrombomodulin was Glu Pro (*Biochemistry,* Vol.26, p.4350, 1987).

Also, Andersen *et al.* have attempted to produce a human thrombomodulin-like substance which corresponds to a moiety of the human thrombomodulin molecule, by means of genetic engineering techniques (International Patent Application WO 88/09811).

In addition, P. W. Majerus *et al.* have developed a cDNA clone of human thrombomodulin by means of genetic engineering techniques and succeeded in expressing a protein molecule having complete amino acid sequence of human thrombomodulin (Japanese Patent Application Kokai No. 63-301791).

Disclosure of the Invention

The inventors of the present invention have isolated a gene of human thrombomodulin precursor from a human cDNA library, prepared various DNA fragments from its partial structures and incorporated these fragments into microorganisms and cells in order to examine biological activities of polypeptides encoded by the DNA fragments. As a result of other series of studies, the present inventors have isolated a thrombomodulin- like substance having a molecular weight of 72 K from human urine (European Patent Publication EP 376251) and have revealed that its structure and activity are different from those of already reported human thrombomodulin molecules. This new substance is hereinafter referred to as "human urine thrombomodulin". The present inventors have prepared DNA fragments, one encoding a polypeptide having the same amino acid sequence of this human urine thrombomodulin and the other fragments encoding derivatives of the polypeptide in which some amino acids of the amino acid sequence were modified by substitution, deletion, addition and the like, incorporated the thus prepared DNA fragments into microorganisms and cells, recovered polypeptides expressed in the host and checked for their biological activities and, as the results, have succeeded in obtaining novel polypeptides each of which having a thrombin binding ability, anticoagulant activity and thrombolytic activity, thereby accomplishing the present invention. These new polypeptides are hereinafter referred to as "recombinant human urine thrombomodulin (ruTM)".

The following describes the present invention in detail.

This invention relates to a novel polypeptide obtained by genetic recombination techniques, having activities similar to human thrombomodulin such as anticoagulant activity and thrombolytic activity, to a DNA fragment which encodes said polypeptide and to a process for the production of said polypeptide by means of recombinant DNA techniques, as well as to an agent for use in the prevention and treatment of hypercoagulability related diseases which comprises said polypeptide as an active ingredient. According to the present invention, there is provided a polypeptide having a thrombin binding ability, anticoagulant activity and thrombolytic activity, which comprises an amino acid sequence represented by the following formula. In the present invention, each amino acid sequence was described using the three letter code started from the N terminus. Amino acid numbers used herein are based on those of the human thrombomodulin reported by Suzuki *et al.* (*EMBO Journal,* Vol.6, p.1891, 1987).

A polypeptide which comprises an amino acid sequence represented by the following formula:

X_1 Glu Pro Gln Pro Gly Gly Ser Gln Cys Val Glu

5 10

His Asp Cys Phe Ala Leu Tyr Pro Gly Pro Ala Thr

15 20 25

```
Phe Leu Asn Ala Ser Gln Ile Cys Asp Gly Leu Arg
                30                  35
Gly His Leu Met Thr Val Arg Ser Ser Val Ala Ala
     40                  45                  50
Asp Val Ile Ser Leu Leu Leu Asn Gly Asp Gly Gly
                55                  60
Val Gly Arg Arg Arg Leu Trp Ile Gly Leu Gln Leu
          65                  70
Pro Pro Gly Cys Gly Asp Pro Lys Arg Leu Gly Pro
 75                  80                  85
Leu Arg Gly Phe Gln Trp Val Thr Gly Asp Asn Asn
                90                  95
Thr Ser Tyr Ser Arg Trp Ala Arg Leu Asp Leu Asn
     100                 105                 110
Gly Ala Pro Leu Cys Gly Pro Leu Cys Val Ala Val
                115                 120
Ser Ala Ala Glu Ala Thr Val Pro Ser Glu Pro Ile
          125                 130
Trp Glu Glu Gln Gln Cys Glu Val Lys Ala Asp Gly
135                 140                 145
Phe Leu Cys Glu Phe His Phe Pro Ala Thr Cys Arg
               150                 155
Pro Leu Ala Val Glu Pro Gly Ala Ala Ala Ala Ala
     160                 165                 170
Val Ser Ile Thr Tyr Gly Thr Pro Phe Ala Ala Arg
                175                 180
Gly Ala Asp Phe Gln Ala Leu Pro Val Gly Ser Ser
          185                 190
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Ala Ala Val Ala Pro Leu Gly Leu Gln Leu Met Cys
195                 200                 205
Thr Ala Pro Pro Gly Ala Val Gln Gly His Trp Ala
            210                 215
Arg Glu Ala Pro Gly Ala Trp Asp Cys Ser Val Glu
    220                 225                 230
Asn Gly Gly Cys Glu His Ala Cys Asn Ala Ile Pro
                235                 240
Gly Ala Pro Arg Cys Gln Cys Pro Ala Gly Ala Ala
        245                 250
Leu Gln Ala Asp Gly Arg Ser Cys Thr Ala Ser Ala
255                 260                 265
Thr Gln Ser Cys Asn Asp Leu Cys Glu His Phe Cys
            270                 275
Val Pro Asn Pro Asp Gln Pro Gly Ser Tyr Ser Cys
    280                 285                 290
Met Cys Glu Thr Gly Tyr Arg Leu Ala Ala Asp Gln
                295                 300
His Arg Cys Glu Asp Val Asp Asp Cys Ile Leu Glu
        305                 310
Pro Ser Pro Cys Pro Gln Arg Cys Val Asn Thr Gln
315                 320                 325
Gly Gly Phe Glu Cys His Cys Tyr Pro Asn Tyr Asp
            330                 335
Leu Val Asp Gly Glu Cys Val Glu Pro Val Asp Pro
    340                 345                 350
Cys Phe Arg Ala Asn Cys Glu Tyr Gln Cys Gln Pro
                355                 360
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Leu Asn Gln Thr Ser Tyr Leu Cys Val Cys Ala Glu
        365                 370
Gly Phe Ala Pro Ile Pro His Glu Pro His Arg Cys
375                 380                 385
Gln Met Phe Cys Asn Gln Thr Ala Cys Pro Ala Asp
            390                 395
Cys Asp Pro Asn Thr Gln Ala Ser Cys Glu Cys Pro
    400                 405                 410
Glu Gly Tyr Ile Leu Asp Asp Gly Phe Ile Cys Thr
                415                 420
Asp Ile Asp Glu Cys Glu Asn Gly Gly Phe Cys Ser
        425                 430
Gly Val Cys His Asn Leu Pro Gly Thr Phe Glu Cys
435                 440                 445
Y1
```

[in this formula, X_1 is a sequence represented by the following formula:

```
Met Leu Gly Val Leu Val Leu Gly Ala Leu Ala Leu
            -15                 -10
Ala Gly Leu Gly Phe Pro Ala Pro Ala
    -5              -1   1
```

or its variation in which optional number or entire amino acids are deleted starting from its N-terminus, and Y_1 is a sequence represented by the following formula:

```
Ile Cys Gly Pro Asp Ser Ala Leu  Z  Arg His
                450                 455
```

[in this instance, Z is Val or Ala]
or its variation in which optional number or entire amino acids are deleted starting from its C - terminus], preferably a polypeptide which comprises the above amino acid sequence in which X_1 is a sequence represented by the following formula:

Ala Pro Ala

1

and Y_1 is a sequence represented by the following formula:

Ile Cys Gly Pro Asp Ser Ala Leu Z Arg

450 455

[in this instance, Z is val or Ala]
or its variation in which optional number or entire amino acids are deleted starting from its C-terminus.

More preferably, a polypeptide which comprises the above amino acid sequence in which X_1 is a sequence represented by the following formula:

Ala Pro Ala

1

and Y_1 is a sequence represented by the following formula:

Ile Cys Gly Pro Asp Ser Ala Leu Z Arg

450 455

[in this instance, Z is Val or Ala]
or a polypeptide which comprises the above amino acid sequence in which X_1 is a sequence represented by the following formula:

Ala Pro Ala

1

and entire amino acids of Y_1 are deleted.

In addition, according to the polypeptide of the present invention, at least one amino acid of the above amino acid sequence may or may not have a sugar chain. The term "sugar chain" as used herein refers to a single sugar or a straight or branched chain of a plurality of sugars which may be in the form of so-called N-glycosidic linkage type or O-glycosidic linkage type. It is known that the activity of thrombomodulin changes depending on the linkage type of sugar chains. For example, in the case of O-glycosidic linkage type sugar chain, Parkinson, J. F. *et al.* have reported recently that human thrombomodulin prepared by means of genetic engineering techniques had a chondroitin sulfate- like sugar chain (*J. Biol. Chem.*, Vol.265, p.12602, 1990). Such a sugar chain-containing polypeptide is also included in the scope of the present invention.

Because of the high technical levels attained in recent years, a part of chemical structure of a polypeptide can be changed easily without altering its activity. Consequently, any polypeptide having an amino acid sequence which has been obtained by partially modifying the aforementioned amino acid sequence by substitution, deletion, addition or the like is also included in the scope of the present invention.

According to the present invention, there is provided a DNA fragment which encodes the aforementioned inventive polypeptide. The DNA fragment of the present invention also includes any fragment having a nucleotide sequence which encodes the aforementioned modified polypeptide of the inventive polypeptide derived by means of substitution, deletion, addition or the like.

The DNA fragment of the present invention may be any fragment, provided that it contains a nucleotide

sequence which encodes the inventive polypeptide, but may preferably contain a nucleotide sequence represented by the following formula. In this instance, nucleotide sequence of the DNA fragment is shown starting from its 5'-end. Also in this instance, A, G, C and T indicate deoxyadenylic acid, deoxyguanylic acid, deoxycytidylic acid and thymidylic acid, respectively.

X_2 GAGCCGC AGCCGGGTGG CAGCCAGTGC GTCGAGCACG 100

ACTGCTTCGC GCTCTACCCG GGCCCCGCGA CCTTCCTCAA 140

TGCCAGTCAG ATCTGCGACG GACTGCGGGG CCACCTAATG 180

ACAGTGCGCT CCTCGGTGGC TGCCGATGTC ATTTCCTTGC 220

TACTGAACGG CGACGGCGGC GTTGGCCGCC GGCGCCTCTG 260

GATCGGCCTG CAGCTGCCAC CCGGCTGCGG CGACCCCAAG 300

CGCCTCGGGC CCCTGCGCGG CTTCCAGTGG GTTACGGGAG 340

ACAACAACAC CAGCTATAGC AGGTGGGCAC GGCTCGACCT 380

CAATGGGGCT CCCCTCTGCG GCCCGTTGTG CGTCGCTGTC 420

TCCGCTGCTG AGGCCACTGT GCCCAGCGAG CCGATCTGGG 460

AGGAGCAGCA GTGCGAAGTG AAGGCCGATG GCTTCCTCTG 500

CGAGTTCCAC TTCCCAGCCA CCTGCAGGCC ACTGGCTGTG 540

GAGCCCGGCG CCGCGGCTGC CGCCGTCTCG ATCACCTACG 580

GCACCCCGTT CGCGGCCCGC GGAGCGGACT TCCAGGCGCT 620

GCCGGTGGGC AGCTCCGCCG CGGTGGCTCC CCTCGGCTTA 660

CAGCTAATGT GCACCGCGCC GCCCGGAGCG GTCCAGGGGC 700

ACTGGGCCAG GGAGGCGCCG GGCGCTTGGG ACTGCAGCGT 740

GGAGAACGGC GGCTGCGAGC ACGCGTGCAA TGCGATCCCT 780

GGGGCTCCCC GCTGCCAGTG CCCAGCCGGC GCCGCCCTGC 820

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AGGCAGACGG GCGCTCCTGC ACCGCATCCG CGACGCAGTC    860
CTGCAACGAC CTCTGCGAGC ACTTCTGCGT TCCCAACCCC    900
GACCAGCCGG GCTCCTACTC GTGCATGTGC GAGACCGGCT    940
ACCGGCTGGC GGCCGACCAA CACCGGTGCG AGGACGTGGA    980
TGACTGCATA CTGGAGCCCA GTCCGTGTCC GCAGCGCTGT   1020
GTCAACACAC AGGGTGGCTT CGAGTGCCAC TGCTACCCTA   1060
ACTACGACCT GGTGGACGGC GAGTGTGTSG AGCCCGTGGA   1100
CCCGTGCTTC AGAGCCAACT GCGAGTACCA GTGCCAGCCC   1140
CTGAACCAAA CTAGCTACCT CTGCGTCTGC GCCGAGGGCT   1180
TCGCGCCCAT TCCCCACGAG CCGCACAGGT GCCAGATGTT   1220
TTGCAACCAG ACTGCCTGTC CAGCCGACTG CGACCCCAAC   1260
ACCCAGGCTA GCTGTGAGTG CCCTGAAGGC TACATCCTGG   1300
ACGACGGTTT CATCTGCACG GACATCGACG AGTGCGAAAA   1340
CGGCGGCTTC TGCTCCGGGG TGTGCCACAA CCTCCCCGGT   1380
ACCTTCGAGT GC  Y2                            1392
```

[in this formula, S is G or C; X_2 is a sequence represented by the following formula:

```
ATGCTTGGGG TCCTGGTCCT TGGCGCGCTG GCCCTGGCCG    40
GCCTGGGGTT CCCCGCWCCC GCA                      63
```

[provided that W is T or A]
or its variation in which optional number or entire nucleotides are deleted in triplets starting from its 5'-end; and Y_2 is a sequence represented by the following formula:

```
ATCTGCGGGC CCGACTCGGC CCTTGYCCGC CAC           1425
```

[provided that Y is T or C]
or its variation in which optional number or entire nucleotides are deleted in triplets starting from its 3'-end].

In addition to the above nucleotide sequence, the DNA fragment of the present invention may have an appropriate promoter and an SD sequence (or a suitable ribosome binding site) bonded to its 5, - end, and if necessary a nucleotide sequence containing a translation initiation codon bonded to the 5, - end and a nucleotide sequence containing a termination codon bonded to the 3'-end.

More preferably, in the nucleotide sequence of the DNA fragment, X_2 is a sequence represented by the following formula:

```
GCWCCCGCA                                      63
```

[in this formula, W is T or A]
and Y_2 is a sequence represented by the following formula:

ATCTGCGGGC CCGACTCGGC CCTTGYCCGC 1422

[in this instance, Y is T or C];
or X_2 is a sequence represented by the following formula:

GCWCCCGCA 63

[in this formula, W is T or A]
and entire nucleotides of Y_2 are deleted.

As it is well known, at least one nucleotide in a gene can be replaced by other nucleotide in accordance with the degeneracy of codon, without changing amino acid sequence of a polypeptide encoded by the gene. In consequence, the DNA fragment of the present invention may have a nucleotide sequence derived from the above inventive nucleotide sequence in which at least one nucleotide has been replaced by other nucleotide in accordance with the degeneracy of codon, especially a nucleotide sequence in which at least one nucleotide has been replaced by other nucleotide in such a way that the resulting codon shows high utilization frequency in a specific host cell when the polypeptide of the present invention is produced making use of genetic engineering techniques.

The DNA fragment of the present invention may be prepared from a natural source or synthesized chemically. The following describes examples of such processes.

In the case of the use of natural source, the DNA fragment of the present invention may be obtained by preparation of a DNA fragment encoding the inventive nucleotide sequence by using natural source such as a cDNA library prepared from cells or tissues containing thrombomodulin mRNA, a commercially available cDNA library or a chromosomal gene and then converting the thus prepared fragment into the inventive fragment.

For the purpose of preparing a cDNA library, mRNA is extracted from human tissues or human cells containing human thrombomodulin mRNA in accordance with a known method (for example, *Molecular Cloning, a laboratory manual,* T. Maniatis *et al.,* Cold Spring Harbor Laboratory, 1982). Next, single-stranded cDNA is prepared using the obtained mRNA as a template followed by the synthesis of double-stranded cDNA from the single stranded cDNA (cf. *Molecular Cloning, a laboratory manual,* cited above; Land's method disclosed in *Nucleic Acid Research,* Vol.9, pp.2251 - 2266, 1981; Okayama- Berg's method in *Mol. Cell. Biol.,* Vol.2, pp.161 - 170, 1982; and Gubler- Hoffman's method in *Gene,* Vol.25, p.263, 1983). The thus obtained double-stranded cDNA fragments are cloned into a plasmid vector such as pBR322, pUC18 or the like or a phage vector such as λ gt10, λ gt11 or the like, and then transformed into *E. coli* or the like to obtain a DNA library.

When a chromosomal gene is used as a source of DNA, chromosomal DNA is extracted from human tissues or human cells, the extracted DNA is digested with appropriate restriction enzymes or by physical means, the digested fragments are cloned into a plasmid or phage vector and then the resulting vector is transformed into *E. coli* or the like to obtain a DNA library.

A DNA fragment encoding the inventive nucleotide sequence is then detected and isolated from the thus obtained DNA library. That is, a plasmid or a phage DNA encoding of the present invention is detected by a usually used means such as hybridization method (*Wallace et al., Nucleic Acid Res.,* Vol.9, p.879, 1981) and then said DNA is isolated from the thus detected plasmid or phage. A DNA or an RNA fragment which has been synthesized in such a manner that it encodes entire or a part of the amino acid sequence of the polypeptide of the present invention, as disclosed herein, may be subjected to radiation labeling to obtain a convenient probe. The radiation labeling may be effected generally by labeling DNA fragment or RNA fragment with ^{32}P, making use of kination, nick translation, random priming or the like method.

The thus isolated DNA fragment from a DNA library by the aforementioned process may be converted into the DNA fragment of the present invention in the following manner. For instance, as a preferred example, the thus isolated DNA fragment from a DNA library is digested by restriction enzymes to obtain desired DNA fragments. Separately from this, a nucleotide sequence which encodes N-terminal or C-terminal region of the polypeptide of the present invention, as well as a termination codon, a restriction enzyme recognition site, a translation initiation codon and the like, are synthesized chemically by a method

which will be described later. After ligating an appropriate synthetic linker to the thus synthesized sequences and codons, they are linked to the DNA fragments obtained above and then inserted into a plasmid or a phage vector as a DNA fragment of interest. When oligonucleotides are synthesized chemically, it is possible to make an appropriate replacement of the nucleotide sequence.

Polymerase chain reaction (to be referred to as "PCR" hereinafter) may also be used as another preferable method. That is, oligonucleotide having nucleotide sequences which encodes N- terminal region, C- terminal region and an intermediate region of the polypeptide of the present invention or, if necessary, these oligonucleotides containing a termination codon, convenient restriction sites, a translation initiation codon and the like are synthesized chemically. Using the thus synthesized oligonucleotide as primers, a DNA fragment isolated from a DNA library by the aforementioned method is subjected to PCR and the DNA fragment of the present invention is obtained. An appropriate replacement of nucleotide also may be introduced to the primers. Alternatively, the aforementioned DNA library may be subjected directly to PCR making use of these primers to amplify and isolated the DNA fragment of the present invention, which are then cloned into an appropriate plasmid or phage vector. The PCR method can be carried out in the light of references or a book (*PCR Protocols, A Guide to methods and applications,* Michael A. I. *et al.,* Academic Press, 1990).

In addition to the aforementioned methods, other commonly used methods may be available, such as the method of Kramer W. *et al.* (*Nucleic Acid Res.,* Vol.12, pp.9441-9465, 1984) and site- directed mutagenesis (*Methods in Enzymology,* Vol.154, pp.350-367, 1988).

On the other hand, when the inventive fragment is prepared by chemical synthesis, a nucleotide sequence of interest is designed and, if necessary, divided into fragments having proper lengths and then corresponding oligomers are synthesized chemically using a full automatic DNA synthesizer (for example, Model 381A manufactured by Applied Biosystems, Inc). If necessary, the thus obtained DNA oligomer may be subjected to phosphorylation of its DNA 5' - end using T4 polynucleotide kinase, followed by annealing. In addition, if necessary, it is possible to clone the resulting DNA fragment into an appropriate vector using T4 DNA ligase.

According to the present invention, there is provided a process for the production of the polypeptide of the present invention which comprises performing at least one step selected from the following steps of:

a) preparing a DNA fragment containing a nucleotide sequence which encodes said polypeptide,

b) inserting said DNA fragment into an expression vector to obtain a recombinant DNA fragment which contains said DNA fragment and is capable of undergoing replication,

c) transforming a host cell with said recombinant DNA fragment to isolate a transformant which can express said polypeptide, and

d) culturing said transformant to allow the transformant to produce said polypeptide and recovering said polypeptide from resulting cultured mixture.

A DNA fragment containing a nucleotide sequence which encodes the polypeptide of the present invention may be obtained by the aforementioned means.

Any vector systems may be used as the expression vector of this process, provided that it is capable of undergoing replication in a host to be used, but preferably a vector which contains a promoter necessary for the expression of the polypeptide in a host and, if required, an SD sequence (or a suitable ribosome binding region) and/or a DNA sequence coding for a signal peptide may be employed. All promoters, SD sequences (or suitable ribosome binding regions) and nucleotide sequences encoding signal peptide which work in host can be used which may be obtained by chemical synthesis or derived from hosts to be used, virus, plasmids, phage and the like.

With regard to the host cells to be used for the introduction of the thus obtained recombinant DNA fragment, suitable cells for the expression of the polypeptide of the present invention may be selected from either eukaryotic cells such as COS cells, CHO cells, yeasts and the like or prokaryotic cells such as *E. coli* , *Bacillus subtilis* and the like, of which COS cells and CHO cells are particularly preferred. It is effective to use a host and an expression vector in such a combination that they can exhibit effective expression of the DNA fragment which encodes the inventive polypeptide. Preferred examples of the combination of host cells with expression vectors include: COS- 7 cells or CHO cells with an expression vector containing the simian virus 40 (SV40) early promoter, with pH β APr-neo containing the human β-actin promoter or with a mammal expression vector derived from pCDL- SR α 296 containing the SR α promoter; and *E. coli* HB101 with an expression vector containing a DNA fragment which encoded a tryptophan promoter and a tryptophan SD sequence.

A host thus transformed with an expression vector may be cultured by generally used means for the culturing of microorganisms or animal cells, in accordance with the procedure disclosed for instance in *Seibutsu Kagaku Kogaku* (or Biochemical Engineering; S. Aiba *et al.,* 1976, Tokyo University Press) or in

Soshiki Baiyo (or Tissue Culture; J. Nakai *et al.*, 1976, Asakura Shoten). The thus produced polypeptide by the transformed host cells is recovered by isolating and purifying it from the cultured mixture. Purification of the polypeptide may be carried out in the light of various generally used means which have been disclosed in many reports and books such as *Seikagaku Jikken Koza* (or Biochemical Experiments; vol.I, Protein Chemistry, 1976, edited by The Japanese Biochemical Society, Tokyo Kagaku Dojin), for instance by using an appropriate combination of purification means selected from dialysis, salting-out, gel filtration, acid precipitation, ion exchange chromatography, affinity chromatography, high performance chromatography, electrophoresis and the like. Preferably, the polypeptide of the present invention may be recovered from the cultured mixture making use of at least one means selected from ion exchange chromatography, affinity chromatography in which thrombin is used as a ligand and gel chromatography.

For example, a cultured mixture containing the polypeptide of the present invention is firstly subjected to desaltation and concentration, for instance making use of an ultrafiltration membrane with a cutoff molecular weight of 30,000. Next, the thus concentrated cultured mixture is adjusted to pH 5 to 10, preferably pH 7.3 ± 0.2, treated at 50 to 70°C for 5 to 45 minutes, preferably at 60 ± 5°C for 15 ± 5 minutes, in order to inactivate proteases, and then applied to a column packed with an anion exchange resin which has been equilibrated to pH 5.5 to 7.5, preferably pH 6.5 ± 0.2. The thus adsorbed active fraction is eluted with an eluent having a pH value of 2 to 4.5, preferably pH 4.0 ± 0.05. The resulting eluate is subjected to desaltation and concentration using an ultrafiltration membrane with a cutoff molecular weight of 30,000. After adjusting to pH 7.5, the thus concentrated eluate is subjected to affinity column chromatography in which thrombin is used as a ligand, the resulting column is washed with a buffer solution containing 0.05 to 0.3 M NaCl, preferably 0.1 ± 0.05 M NaCl, active fraction is eluted with an eluent containing 0.9 to 2.0 M NaCl, preferably 1.0 ± 0.05 M NaCl. After subjecting to desaltation and concentration, the thus concentrated eluate is again subjected to affinity column chromatography in which thrombin is used as a ligand, the resulting column is washed with buffer solution containing 0.3 to 0.8 M NaCl, preferably 0.7 ± 0.1 M NaCl, and then active fraction is eluted with an eluent containing 0.9 to 2.0 M NaCl, preferably 1.0 ± 0.05 M NaCl. Thereafter, the thus eluted polypeptide is subjected to desaltation and concentration and then to gel filtration column chromatography, to obtain an active fraction corresponding to the polypeptide of the present invention from which the inventive polypeptide can be obtained in a purified form. Alternatively, the polypeptide of the present invention may be obtained in a purified form, by subjecting the eluted fraction from the aforementioned affinity column to desaltation and concentration and then applying the concentrated fraction to SDS- PAGE under non- reducing condition. The thus obtained polypeptide of the present invention can be made into a pharmacologically acceptable form by inactivating viruses through heat treatment at 60 ± 2°C for 10 hours.

Examples of the anion exchange resin eligible for use in the aforementioned purification process include DEAE cellulose, DEAE Sepharose, DEAE Cellulofine and the like, while the aforementioned affinity column in which thrombin is used as a ligand may be prepared by binding thrombin to a carrier such as cellulose, agarose, dextran or the like using cyanogen bromide and then treating the resulting resin with diisopropyl fluorophosphate (DIP), phenylmethanesulfonyl fluoride or the like. As a resin for use in gel filtration, Sephacryl S-200, Sephacryl S-300, Sephadex G150 or the like may be effective.

By applying the procedure described above, the polypeptide of the present invention can be obtained in a purified form. By the use of the same procedure, a different substance having similar properties can also be obtained.

The following describes actions and properties of the polypeptide of the present invention.

(Experimental Example 1) Affinity for thrombin (anti-thrombin action)

a) When treated chromatographically using DIP-thrombin agarose, a pKCR- TM- Val- originated recombinant human urine thrombomodulin (to be referred to as "ruTM-Val" hereinafter) prepared in Example 3 and another recombinant human urine thrombomodulin (to be referred to as "ruTM-Ala" hereinafter) prepared in Example 6-(2) are adsorbed by thrombin with almost 100% accuracy.

b) A 100 µl portion of bovine thrombin solution (1 U/ml, manufactured by Mochida Pharmaceutical Co., Ltd.) is mixed with 100 µl of a solution containing ruTM-Val or ruTM-Ala, the thus mixed solution is incubated at 37°C for 30 minutes and then the resulting solution is mixed with 100 µl of human fibrinogen solution (2 mg/ml, manufactured by Sigma Chemical Co.) to measure coagulation time using a coagulometer (manufactured by Amelung Co. Ltd.).

The results are shown in Table 1.

Table 1

Drugs	Concentration(OD_{280})	Coagulation time(seconds)
Control	—	37.8
ruTM-Val	0.01	> 500
ruTM-Ala	0.01	> 500

As is evident from these results, ruTM-Val and ruTM-Ala have functions to bind to thrombin and strongly inhibit its coagulation activity.

Table 2 shows data adduced from Japanese Patent Application Kokai No. 62- 169728 on the coagulation time measured using a thrombomodulin- like substance purified from human placenta.

Table 2

Drug	Concentration (OD_{280})	Coagulation time (seconds)
Control	—	35.8
Human placenta thrombomodulin-like substance	0.42 0.84	62.3 109.9

In addition, according to the just cited publication, there is a description that this human placenta thrombomodulin-like substance has two times or more higher activity than the existing human thrombomodulin, thus leading to a conclusion that, from the comparison of the results shown in Tables 1 and 2, ruTM-Val and ruTM-Ala have stronger anti-thrombin activity than the existing human thrombomodulin.

(Experimental Example 2) Protein C activating ability

Protein C activating ability is measured in the presence of thrombin, using a synthetic substrate Boc-Leu-Ser - Thr - Arg - MCA (manufactured by Peptide Research Institute, Protein Research Foundation). That is, 60 μl of 0.1 M Tris-HCl buffer (pH 7.5) is mixed with 20 μl of a 10 U/ml solution of bovine thrombin (manufactured by Mochida Pharmaceutical Co., Ltd.), 10 μl of a solution containing ruTM-Ala obtained in Example 6- (2) and a mutation type recombinant human urine thrombomodulin (to be referred to as "DEL 10" hereinafter) in which 10 amino acid residues are deleted from the C- terminus of the human urine thrombomodulin (0.1 to 10 μg/ml in total), and 10 μl of 500 μg/ml solution of human protein C (American Diagnostica, Inc.), in that order. After incubation at 37 °C for 30 minutes, the resulting reaction mixture is mixed with 150 μl of a mixture solution consisting of the same volume of 1 U/ml human antithrombin (manufactured by The Green Cross Corporation) and 10 U/ml heparin (manufactured by Mochida Pharmaceutical Co., Ltd.), followed by additional incubation at 37 °C for 15 minutes. Next, the resulting reaction mixture is mixed with 250 μl of 0.1 mM solution of the aforementioned synthetic substrate and incubated at 37 °C for 10 minutes to complete the reaction which is then stopped by the addition of 500 μl of 20% acetic acid solution. Thereafter, the reaction solution is subjected to the measurement of fluorescence strength using an fluorophotometer (Hitachi, Ltd.) at an excitation wave length of 380 nm and an emission wave length of 460 nm. In this instance, human placenta thrombomodulin purified from human placenta in accordance with the procedure shown in Reference Example was used as a positive control. As shown in Table 3, protein C activating abilities of ruTM-Ala and DEL 10 calculated from the fluorescence strength are markedly high in the presence of thrombin in comparison with that of human placenta thrombomodulin.

Table 3

	Activity *1
ruTM-Ala	3.8
DEL 10	4.1
Human placenta thrombomodulin	1.0

*1: Relative activity when the activity of human placenta thrombomodulin is defined as 1.

(Experimental Example 3) Anticoagulant activity

A 100 μl portion of a citric acid-added platelet poor plasma sample obtained from a healthy person is mixed with 10 μl of a solution containing ruTM-Val or ruTM-Ala (10 - 100 μg/ml), the thus prepared mixture is incubated at 37 °C for 2 minutes and then the reaction solution is mixed with 100 μl of human thrombin (manufactured by the Green Cross Corporation, 2 U/ml) to measure coagulation time and to find strong function of ruTM-Val and ruTM-Ala to prolong blood coagulation time.

(Experimental Example 4) Acute toxicity in mouse

When ruTM- Val or ruTM-Ala was administered by intravenous injection to 5 individuals of ddY male mouse and observed for 7 days, no case of significant toxicity or death was found within the effective dose.

(Experimental Example 5) Solubility

At room temperature, ruTM- Val and ruTM- Ala dissolved in distilled water to a concentration of at least 30 mg/ml.

In addition, when intravenously administered *in vivo*, the water soluble ruTM shows excellent DIC improving function in comparison with the slightly soluble placenta thrombomodulin which has a phospholipid binding ability.

Thus, since the polypeptide of the present invention has strong thrombin binding ability, anticoagulant activity and thrombolytic activity and has low toxicity as clear from the foregoing description and experiments, the inventive polypeptide may be used efficiently for the prevention and treatment of hypercoagulability-related diseases such as DIC, various types of thrombosis, peripheral vessel obstruction, myocardial infarction, cerebral infarction, transient cerebral ischemic attack, gestational toxicosis, hepatic insufficiency, renal insufficiency and the like.

The polypeptide of the present invention can be made into pharmaceutical preparations, preferably injections, suitable for use in efficient administration to patients, by mixing it with proper carrier or medium such as sterile water, physiological saline, a plant oil, a non-toxic organic solvent or the like generally used as drugs and, if necessary, further with a filler, a coloring agent, an emulsifying agent, a suspending agent, a stabilizer, a preservative or the like. When the polypeptide of the present invention is used as an injection, it may be administered to each patient at a time or continuously by dividing its daily dose into 1 to 6 times. Daily dose of the polypeptide of the present invention may be in the range of from 0.05 to 500 mg potency, preferably from 0.1 to 10 mg potency, as a calculated value in terms of the potency of rabbit lung thrombomodulin, though the dose may be changed suitably depending on each patient's age, weight, symptoms and the like.

In addition, the polypeptide of the present invention can be used by binding or adsorbing it to the surface of medical devices such as artificial blood vessels, artificial organs, catheters and the like, making use of a cross-linking agent or the like. By such a treatment, blood coagulation on the surface of medical devices can be prevented.

Best Mode for the Practice of the Invention

Examples of the present invention are given below by way of illustration and not by way of limitation. Abbreviations used herein are based on idiomatical expressions used in this field of science.

Experiments related to genetic recombinantion DNA techniques were carried out, unless otherwise noted, in the light of books including "Maniatis T. *et al, Molecular Cloning,* Cold Spring Harbor Laboratory, 1982" and "S. Kobayashi, *Handbook for Gene Manipulation Experiments,* JATEC Publishers, 1985" and instructions attached to purchased reagents and devices. Also, unless otherwise noted, restriction enzymes used in the following experiments were purchased from Takara Shuzo Co., Ltd. or from New England Biolabs, Inc.

A high ruTM-Ala expressing strain, TMM-B1C, used in the following examples has been deposited on June 25, 1991, in Fermentation Research Institute, Agency of Industrial Science and Technology, and has been assigned the designation as FERM BP-3463.

Example 1

Cloning of thrombomodulin cDNA and construction of expression plasmid

(1) Cloning of thrombomodulin cDNA

The oligonucleotide probe shown in Fig. 1 was prepared using a DNA synthesizer (already mentioned) based on an amino acid sequence,

Glu His Asp Cys Phe Ala,

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which is a part of the N-terminal amino acid sequence of human urine thrombomodulin isolated and purified from human urine. The thus synthesized oligonucleotide was purified using OPC column (Applied Biosystems, Inc.) and its 5'-end was labeled using T4 polynucleotide kinase (Takara Shuzo Co., Ltd.) and [γ - ^{32}P] ATP (Amersham). Next, the resulting reaction solution was applied to Sephadex G- 25 column (Pharmacia) to separate the labeled oligonucleotide probe from [γ-^{32}P] ATP for use in the following procedure as a probe.

Total RNA was prepared from about 20 g portion of human placenta by means of guanidinium isothiocyanate extraction. A 10 mg of the thus extracted total RNA was subjected twice to oligo (dT)-cellulose chromatography (type 7, Pharmacia) to obtain about 90 μg of purified poly A$^+$ RNA, followed by cDNA construction using the thus obtained poly A$^+$ RNA. That is, double-stranded cDNA was prepared (using a cDNA synthesizing system, Amersham) from 20 μg of the poly A$^+$ RNA using oligo dT as a primer by the method of Gubler and Hoffman (Gubler, U. and Hoffman, B.J., *Gene,* Vol.25, p.263, 1983). The thus prepared cDNA was subjected to methylation using *Eco*RI methylase and then *Eco*RI linkers were linked. After digestion with *Eco*RI, the free linker and DNA fragments less than 500 bp were removed by gel filtration (BioGel A50m, Bio-Rad Laboratories). The resulting DNA fragment was cloned into a phage vector λ gt11 (Amersham) to prepare a cDNA library, with an efficiency of about 90% and containing about 2 x 10^6 independent clones. Phage particles in the thus prepared λ gt11 library were plated on *E. coli* strain Y1090 as the host in the usual way with such an inoculum size that plaques were formed about 5 x 10^3 per plate having a diameter of 9 cm. The thus formed plaques were transferred on a nylon filters (Hybond-N, Amersham), and the resulting filters were put on filter paper soaked with 1.5 M NaCl/0.5 M NaOH solution for 5 minutes and then with 1.5 M NaCl/0.5 M Tris-HCl buffer (pH 8.0) for 5 minutes to denature DNA. Next, the thus treated nylon filters were washed with 0.36 M NaCl/20 mM sodium phosphate (pH 7.4)/2 mM EDTA (pH 7.4) solution and then air-dried. After fixing the DNA on the filters by ultraviolet ray irradiation, the resulting filters were washed with a 0.1% SDS/x 0.1 SSC solution (SSC: x 1 concentration; 150 mM NaCl/15 mM sodium citrate, pH 7.0) at 65 °C for 1 hour. The thus DNA-fixed filters were subjected to pre-hybridization at 65 °C for 6 to 24 hours in a solution of x 6 SSC/50 mM sodium phosphate buffer (pH 6.8)/x 1 Denhardt solution/100 μg/ml denatured salmon sperm DNA, followed by overnight hybridization at 37 °C in the same solution supplemented with about 10^6 cpm/ml of the aforementioned 5'-end labeled oligonucleotide. The filters were washed with x 6 SSC for 5 to 30 minutes at 4 °C , 37 °C and 42 °C in that order, air-dried and then subjected to autoradiography.

By checking about 3 x 10^5 plaques through the above procedure, a total of 23 clones showing positive reaction with the probe were isolated. After subjecting each of the thus isolated phage clones to plaque formation, the above hybridization procedure was repeated to obtain 9 clones which showed the positive signal again and from which phage DNA samples were collected. When digested with a restriction enzyme,

*Eco*RI, about 0.7 to 2.5 kb inserted DNA were found in the λ gt11. A restriction map of the largest 2.5 kb inserted DNA is shown in Fig. 2. Two DNA fragments obtained by cleavage with *Eco*RI and *Pst*I of the 2.5 Kb inserted DNA were isolated and subcloned into an M13 phage, mp18 or mp19, between *Eco*RI and *Pst*I cloning site in the usual way to prepare single- stranded phage DNA and were sequenced by a DNA sequencer (370A, Applied Biosystems, Inc.). As the results, a sequence corresponding to the N - terminal sequence of human thrombomodulin was found in an amino acid sequence deduced from the nucleotide sequence of an *Eco*RI/*Pst*I DNA fragment of about 0.4 kb, which confirmed that the cloned cDNA is of human urine thrombomodulin. Figs. 3(a) to 3(m) show results of the nucleotide sequence of the 2.5 kb DNA fragment.

(2) Construction of recombinant human urine thrombomodulin expression vector

Construction of expression plasmid for use in mammalian cells (Fig. 4(a) - Fig. 4(b))

The 2.5 kb thrombomodulin cDNA was digested with *Eco*RI and subjected to agarose gel electrophoresis, and DNA fragments isolated from the gel were subcloned into plasmid pUC118. Plasmid DNA thus prepared was digested with *Eco*RI and then the 3' recessed termini were filled using T4 DNA polymerase (Takara Shuzo Co., Ltd.). The *Bam*HI linkers (Takara Shuzo Co., Ltd.) were connected to the blunt-ended termini using a ligation kit (Takara Shuzo Co., Ltd.), and the resulting DNA fragment was double-digested with *Bam*HI and *Kpn*I, followed by electrophoresis to isolate a DNA fragment of about 1.5 kb from the gel.

Two synthetic oligonucleotide linkers (each linker was prepared from a set of a 49 mer and a 53 mer complementary oligonucleotide) as shown in Fig. 5(a) were obtained using the aforementioned DNA synthesizer, each linker starting from the *Kpn*I site of the human urine thrombomodulin cDNA, encoding a C- terminal amino acid sequence (Leu Ala Arg) of human urine thrombomodulin and ending just after the terminal sequence with a terminal codon and *Bam*HI site. In this case, purification of each single- stranded oligonucleotide was carried out using reverse phase HPLC (C8 column, AQUAPORE RP- 30, Applied Biosystems, Inc.). The 49 mer oligonucleotide was subjected to 5'-end phosphorylation using T4 polynucleotide kinase (already mentioned) and then annealed with the 53 mer oligonucleotide.

Next, the linker was ligated with previously prepared *Bam*HI/*Kpn*I fragment of about 1.5 kb thrombomodulin cDNA (already mentioned), and digested with *Bam*HI and then, the digested DNA fragments were subjected to agarose gel electrophoresis to isolate a 1.6 Kb DNA fragment. On the other hand, an expression vector in mammalian cells, pKCR (O'Hara, K. *et al., Proc. Natl. Acad. Sci.,* USA, Vol.78, p.1527, 1981), was digested with *Bam*HI and then treated with a phosphatase (Takara Shuzo Co., Ltd.) to obtain a linear DNA fragment which was subsequently subjected to ligation (already described) with the 1.6 kb DNA fragment to prepare human urine thrombomodulin expression plasmids, pKCR-TM-Ala and pKCR-TM-Val, in mammalian cells.

Construction of expression plasmid in *E. coli* (Fig. 6(a) - Fig. 6(b))

Each of the aforementioned plasmids, pKCR-TM-Ala and pKCR-TM-Val, was double-digested with *Bam*HI and *Sma*I, and the resulting 1.3 kb DNA fragment was isolated by agarose gel electrophoresis. Next, an oligonucleotide linker consisting of 69 mer and 67 mer synthetic oligonucleotides as shown in Fig. 5(b) was prepared in the same manner as described in the foregoing. The 67 mer oligonucleotide was subjected to phosphorylation using a nucleotide kinase and then annealed with the 69 mer oligonucleotide, the resulting linker was ligated with the aforementioned 1.3 kb DNA fragment, and was double-digested with *Sma*I and *Bam*HI. On the other hand, plasmid pM450 (Kanamori, T. *et al., Gene,* Vol.66, pp.295 - 300, 1988) was double-digested with *Bam*HI and *Nde*I, and were subjected to agarose gel electrophoresis to isolate a DNA fragment of about 3.2kb. The thus prepared 3.2 kb DNA fragment was ligated with each of the two linker - connected thrombomodulin cDNA fragments obtained above to prepare plasmids pM450-TM-Ala and pM450-TM-Val for the expression of recombinant human urine thrombomodulin in *E. coli.*

Example 2

Cloning of thrombomodulin cDNA and construction of expression plasmid

(1) Cloning of thrombomodulin cDNA

A single-stranded cDNA was prepared from 10 μg of the poly A^+ RNA derived from human placenta obtained in Example 1- (1) using an oligo dT primer and a reverse transcriptase (Takara Shuzo Co., Ltd.) as usual.

Separately from this, a total of 6 oligonucleotides (Fig. 7) were prepared using a DNA synthesizer (already mentioned), each of which corresponding to the nucleotide sequence encoded a part of the human urine thrombomodulin cDNA fragment obtained in Example 1-(1), with its 5'-end having a recognition site of a restriction enzyme selected from *Sal*I, *Bam*HI, *Eco*RI, *Hind*III or *Pst*I. In this instance, each of the S1, S2 and S3 oligonucleotides corresponds to a part of "+" strand of the human urine thrombomodulin, while each of the A1, A2 and A3 oligonucleotides corresponds to a part of "-" strand. Also in this instance, *Xho*I site was introduced in the S3 oligonucleotide by means of silent mutation. Also the A3 oligonucleotide contains a DNA sequence which corresponds to a termination codon. The thus synthesized thrombomodulin specific oligonucleotide primers were purified using OPC column (already mentioned).

Next, PCR was carried out using the single-stranded cDNA as a template and the chemically synthesized oligonucleotides as primers to obtain human urine thrombomodulin cDNA by dividing it into three parts. That is, 100 μl of a reaction solution consisting of a 10 mM Tris- HCl (pH 8.3)/50 mM KCl/1.5 mM $MgCl_2$/0.01% gelatin solution containing about 50 ng of the single-stranded cDNA, 0.8 μg of each primer (S1 and A1) and 2.5 units of a thermostable DNA polymerase (Perkin - Elmer Cetus) was applied to Thermal Cycler (Perkin-Elmer Cetus) and PCR was carried out under conditions of: annealing, 55 °C for 2 minutes; synthesis of complementary chain, 72 °C for 3 minutes; thermal denaturation, 94 °C for 1 minute; and cycle numbers, 30. After purification by phenol•chloroform extraction and ethanol precipitation, amplified DNA fragment I having a size of about 450 bp was obtained. The PCR procedure was repeated in the same manner except that S2 and A2 or S3 and A3 were used as primers to obtain DNA fragment II of about 650 bp and DNA fragment III of about 350 bp. The thus prepared fragments I, II and III were digested with *Sal*I/*Bam*HI, *Hind*III/*Sca*I and *Pst*I/*Bam*HI respectively and subcloned into pUC118 in the usual way to obtain pUC118-FI, pUC118-FII and pUC118-FIII.

The three DNA fragments of the human urine thrombomodulin cDNA thus obtained by PCR were connected one another in the following manner to construct a cDNA fragment which encodes a signal peptide and the whole mature protein supplemented with termination codon to its 3'-end.

First, pUC118- FI was digested with *Hind*III and *Bam*HI, and the digested products were subjected to agarose gel electrophoresis in the usual way to isolate a DNA fragment having a size of about 450 bp. The thus isolated DNA fragment was further digested with *Dde*I and the digests were subjected to purification to isolate a DNA fragment, FI, with cohesive end of *Hind*III and DdeI in its 5'-end and 3'- end. In the same manner, another DNA fragment, FII, which has a size of about 650 bp with cohesive end of *Dde*I and *Sal*I in its 5'-end and 3'-end, was obtained by subjecting the pUC118- FII to digestion with *Hind*III and *Sal*I, separation of the resulting digests, digestion with *Dde*I and purification of the fragment of interest. As well as still another DNA fragment, FIII, which has a size of about 350 bp with cohesive end of *Xho*I and *Eco*RI in its 5'-end and 3'-end, was also obtained by subjecting the pUC118-FIII to digestion with *Xho*I and *Eco*RI, separation of the resulting digests and purification of the fragment of interest. Next, the fragments FI, FII and FIII were ligated into *Hind*III/*Eco*RI cloning site of pUC118 to obtain a plasmid pUC-TM. (The construction process is shown in Fig. 8.) The cDNA of interest was subcloned in the usual way into M13 phage, mp18 or mp19, single-stranded DNA fragment was prepared in order to determin oligonucleotide sequence by a DNA sequencer (already mentioned), it was confirmed that this cDNA encoded the human urine thrombomodulin. The results of the nucleotide sequence determination were shown in Fig. 9(a) - Fig. 9(b).

(2) Construction of recombinant human urine thrombomodulin expression vector

The plasmid pUC - TM containing human urine thrombomodulin cDNA prepared in Example 2-(1) was digested with *Sal*I and *Bam*HI, and a DNA fragment of about 1.4 Kb was isolated and purified in the usual way making use of agarose gel electrophoresis. The thus prepared fragment was inserted into a *Sal*I-*Bam*HI cloning site of an expression vector for mammalian cells pH β APr-1-neo (P. Gunning *et al., Proc. Natl. Acad. Sci.,* USA, Vol.84, p.4831, 1987), to construct a vector LK444-TM for the expression of recombinant thrombomodulin. Next, a plasmid pAdD26SV (A) (R. J. Kaufman *et al., Mol. Cell. Biol.,* Vol.2, p.1304, 1982) which contains a gene coding for dihydrofolate reductase (to be referred to as "DHFR" hereinafter) was digested with *Bgl*I and the recessed termini were filled using T4 DNA polymerase (already mentioned), and the fragment was digested with *Eco*RI and then subjected to agarose gel electrophoresis in the usual way to isolate and purify a DNA fragment of about 3 Kb containing the DHFR gene. Next, the expression vector LK444-TM obtained above was digested with a *Aat*II and the recessed termini were filled using T4 DNA polymerase (already mentioned), and the fragment was digested with *Eco*RI and then subjected to agarose gel electrophoresis in the usual way to isolate and purify a DNA fragment of about 9 kb. Thereafter, the thus prepared DNA fragment was ligated with the previously prepared DHFR gene-containing DNA fragment in

the usual way to construct LK444-TM-DHFR. (Fig. 10(a) - Fig. 10(b))

Next, pUC-TM was digested with *Sal*I and *Eco*RI, and subjected to agarose gel electrophoresis in the usual way to isolate and purify a DNA fragment having a length of about 1.4 kb. Together with a *Pst*I-*Sal*I linker (5'-TCGATGCA-3') which has been prepared by a DNA synthesizer (already mentioned) and purified by OPC column (already mentioned), the thus obtained DNA fragment was ligated into a *Pst*I/*Eco*RI cloning site of an expression vector for mammalian cells, pCDL- SR α 296 (Y. Takebe *et al., Mol. Cell. Biol.,* Vol.8, p.466, 1988), to construct a human urine thrombomodulin expression vector, pCDSR α-TM. Next, the thus constructed vector was digested with *Sal*I and *Cla*I, the recessed termini were filled using T4 DNA polymerase (already mentioned) and then subjected to agarose gel electrophoresis in the usual way to isolate and purify a DNA fragment containing the human urine thrombomodulin cDNA. On the other hand, the aforementioned LK444-TM-DHFR was digested with *Eco*RI and *Nde*I, the recessed termini were filled using T4 DNA polymerase (already mentioned) and then subjected to agarose gel electrophoresis in the usual way to isolate a purify DNA fragment containing the DHFR gene. Thereafter, the thus prepared DNA fragment was ligated with the previously prepared DNA fragment containing the human urine thrombomodulin cDNA in the usual way to construct pCDSR α-TM-DHFR. (Fig. 11(a) - Fig. 11(b))

Example 3

Expression of thrombomodulin

Each of the plasmids pKCR-TM-Ala and pKCR-TM-Val prepared in Example 1 was transfected into COS- 7 cells (ATCC No. CRL1651) by means of DEAE dextran method to express recombinant thrombomodulin. That is, semiconfluent COS-7 cells prepared in advance were transfected with the plasmid DNA at a ratio of about 1 μg DNA per about 2 x 10^5 cells in accordance with the method of Lauren et al. (Lauren, M., *Proc. Natl. Acad. Sci.,* USA, Vol.78, p.7575, 1981). The thus treated cells were cultured for 3 days using Dulbecco's modified Eagle's medium (to be referred to as "D-ME medium" hereinafter) which has been supplemented with 0.01% albumin, followed by recovering of culture supernatant to obtain a crude recombinant human urine thrombomodulin solution. Transfection was carried out in the same manner and a 10 liter portion of the resulting culture filtrate was subjected to desalting and concentration making use of an ultrafiltration membrane of 30,000-molecular-weight cutoff.

After adjusting to pH 7.3, the concentrated culture filtrate was treated at 60°C for 15 minutes. The resulting sample was applied to a column packed with 300 ml DEAE cellulose (Whatman) which has been equilibrated with phosphate buffer in advance, the column was washed with 750 ml of the same buffer used for the equilibration, and the thus adsorbed active fraction was eluted with acetate buffer (pH 4.0).

The eluate was concentrated using an ultrafiltration membrane of cutoff molecular weight of 30,000, adjusted to pH 7.5 with 2 M NaOH and then applied to a 2.5 ml of DIP-thrombin-agarose column which has been equilibrated with 0.02 M Tris-HCl buffer (pH 7.5) containing 0.1 M NaCl, 1 mM benzamidine hydrochloride and 0.5 mM $CaCl_2$, thereby adsorbing the active fraction. Next, the column was washed with 25 ml of the same buffer used for the equilibration and the active fraction was then eluted with 0.02 M Tris-HCl buffer (pH 7.5) containing 1 M NaCl, 1 mM benzamidine hydrochloride and 0.5 mM EDTA. The eluate was dialyzed against the same buffer as used in the equilibration and then subjected to purification by means of DIP-thrombin-agarose column chromatography in the same manner as described above.

The resulting eluate was concentrated using an ultrafiltration membrane of cutoff molecular weight of 30,000 and then subjected to gel filtration using a column packed with 500 ml Sephacryl S-300 (Pharmacia Fine Chemicals) which has been equilibrated in advance with 0.01 M phosphate buffer (pH 7.0) containing 0.14 M NaCl, thereby recovering the active fraction of interest.

By carrying out the above production process, about 0.5 mg of purified recombinant human urine thrombomodulin was obtained from each of the culture filtrates derived from pKCR-TM-Ala and pKCR-TM-Val. Each of the thus purified recombinant human urine thrombomodulin showed a single band by non-reduced SDS-PAGE. When examined, both showed high activities.

After subjecting 300 μg of each of the polypeptides of the present invention to reductive carboxymethylation in accordance with the method of C. H. Hirs *et al.* (*Methods in Enzymol.,* Vol. 11, p.199, 1967) and then to desalting, N-terminal amino acid sequences of the thus treated samples were determined using gas phase protein sequencer (Applied Biosystems, Inc., model 470A), and their C-terminal amino acid sequences were analyzed by means of carboxypeptidase method (*Biochem. Biophys. Acta,* Vol.397, p.443, 1975). As the results, the N-terminal and C-terminal amino acid sequences of these two polypeptides coincided with those of the 72 K human urine thrombomodulin. In other words, amino acid sequence of the polypeptide obtained from the pKCR- TM- Ala- originated culture filtrate was,

N-terminal: Ala Pro Ala Glu Pro Gln

1 5

C-terminal: Leu Ala Arg

455

and amino acid sequence of the polypeptide obtained from the pKCR-TM-Val-derived culture filtrate was as follows.

N-terminal: Ala Pro Ala Glu Pro Gln

1 5

C-terminal: Leu Val Arg

455

Example 4

Expression of human urine thrombomodulin

The plasmid pCDSR-α-TM-DHFR constructed in Example 2 was transfected into CHO cells by means of electroporation (the method reported by D. Zerbib *et al. in Biochem. Biophys. Res. Comm.,* Vol.129, p.611, 1985, was slightly modified) in the following manner to express recombinant human urine thrombomodulin.

That is, CHO DXB11 cells (Urlaub, G. and Chasin, L. A., *Proc. Natl. Acad. Sci.,* Vol. 77, p.4216, 1980) were cultured at 37°C for 2 days in 5% CO_2-95% air using Ham's F12 (Flow Laboratories, Inc.) containing 10% fetal bovine serum (Nippon Bio-Supply Center Co., Ltd.) (to be referred to as "medium-①" hereinafter), dispersed by trypsin- EDTA treatment and then suspended in 50 ml of fresh medium-①. The thus prepared cell suspension was centrifuged at 1,000 r.p.m. for 5 minutes using a refrigerated centrifuge (Kokusan Enshinki Co., Ltd.). After discarding the supernatant, the resulting cells were suspended in 50 ml of a sucrose-containing phosphate buffer (540 mM sucrose/7.0 mM sodium dihydrogenphosphate 12 H_2O/4.2 mM magnesium chloride, pH 7.4) and centrifuged at 1,000 r.p.m. for 5 minutes. After repeating the above suspension step in the sucrose-containing phosphate buffer and subsequent centrifugation step, the resulting cells were suspended in the sucrose-containing phosphate buffer to a density of 1×10^7 cells/ml, and 0.4 ml of the thus prepared cell suspension was transferred in a cuvette for an electroporation apparatus, Gene Pulser TM (BIO-RAD). To the cuvette was further added 0.4 ml of plasmid pCDSR-α-TM-DHFR which has been prepared to a concentration of 50 μg/ml of the sucrose-containing phosphate buffer. The resulting mixture in the cuvette was allowed to stand for 15 minutes in an ice bath and then subjected to electroporation using Gene Pulser. Thereafter, the thus treated cells in the cuvette were allowed to stand for 10 minutes in an ice bath and then made into a cell suspension of 1×10^4 cells/ml using the medium-①. 10 ml of the thus prepared cell suspension was transferred in a culture dish of 10 cm in diameter and cultured at 37°C in 5% CO_2-95% air. Two days after the culture, medium in the culture dish was removed and the culture was continued by supplying the dish with 10 ml of MEM α (-) (contains no ribonucleosides or deoxyribonucleosides, manufactured by GIBCO) containing 10% of heat inactivated and dialyzed fetal bovine serum (already mentioned) (to be referred to as "medium-②" hereinafter). The culture was continued by replacing the medium-② with fresh one every 2 to 4 days, and single colonies consisting of 100 to 200 cells were isolated after 16 or 19 days of the culture by means of penicillin cup method. The collected cells were transferred to a 96 well multi-dish (A/S Nunc) and cultured using the medium-②. Each of the thus obtained clones, when it grew into proper level, was cultured again by changing the culture dish. During the culture process, a portion of the cells were cultured in a serum-free medium and the amount of

recombinant thrombomodulin in the resulting culture supernatant was measured in the following manner to evaluate recombinant thrombomodulin productivity of each clone. That is, 3 ml cell suspension adjusted to 4.2 x 10^4 cells/ml using the medium-② was poured in a culture dish of 35 mm in diameter and cultured at 37°C for 3 days in 5% CO_2-95% air. Next, after removing the culture medium, the cultured cells were washed with PBS-Tween and cultured again using 3 ml of MEM α (-) containing 5 KIU/ml of aprotinin (Repulson, Mochida Pharmaceutical Co., Ltd.) at 37°C for 2 days in 5% CO_2-95% air to measure biological activity in the resulting culture supernatant. In this way, a clone showing a high activity was selected as a high expression strain of recombinant thrombomodulin. In addition, the thus selected recombinant thrombomodulin high expression strain was adjusted to 1 x 10^4 cells/ml using the medium-② which has been supplemented with 20 nM methotrexate (Lederle Japan) (to be referred to as "MTX" hereinafter), and 10 ml of the thus prepared cell suspension was poured in a culture dish of 10 cm in diameter and cultured at 37°C in 5% CO_2-95% air. Thus obtained resistant cells to 20 nM MTX were subjected to cloning making use of penicillin cup method, and recombinant thrombomodulin productivity of each clone was evaluated to select a recombinant thrombomodulin high expression strain. The concentration of expression of the thus selected high expression strain, TMM-B1C, was 1.3 μg/ml.

The recombinant thrombomodulin in the culture supernatant was recovered and purified in accordance with the procedure of Example 3, and its N-terminal and C-terminal amino acid sequences were determined also in accordance with the procedure of Example 3. As the results, these sequences were confirmed as follows.

N-terminal: Ala Pro Ala Glu Pro Gln

1 5

C-terminal: Leu Ala Arg

455

Example 5

Expression in *E. coli*

E. coli strain HB101 transformed with the plasmid pM450-TM-Ala or pM450-TM-Val prepared in Example 1 were cultured overnight in 5 ml of L-broth containing 100 μg/ml of ampicillin (to be referred to as "Ap" hereinafter). The resulting culture broth was inoculated into 50 volumes of M9CA medium containing 100 μg/ml Ap and 50 μg/ml tryptophan and cultured at 37°C for about 3 hours until the cell growth reached its late log phase, followed by the addition of 3 β-indoleacrylic acid (Wako Pure Chemical Industries, Ltd.) to a final concentration of 10 μg/ml and subsequent culturing for 3 to 5 hours. The thus cultured cells were recovered using a centrifuge (MR-15, Tomy Seiko Co., Ltd.) and washed with physiological saline, and the resulting precipitate was suspended in a 2% sodium dodecyl sulfate/1 mM EDTA/10 mM Tris-HCl (pH 7.4) solution in an amount equivalent to 1/10 volume of the culture broth to disperse the cells and then lysed by heat treatment at 90°C for 5 minutes. Thereafter, insoluble materials in the lysate were removed by a centrifuge (already mentioned) at 15,000 r.p.m. for 10 minutes, and the resulting supernatant was dialyzed against PBS to obtain a lysate sample.

Two lysate samples obtained in this manner were checked for their reactivity with anti-human urine thrombomodulin antibody. That is, each well of a flat bottom 96 well microtiter plate (Immulom- 600, Greiner, Inc.) was charged with 100 μl of anti-human urine thrombomodulin antibody (obtained by sensitizing a rabbit with 72 K human urine thrombomodulin prepared from urine and purifying the resulting serum by ammonium sulfate precipitation and DEAE-Sepharose column) which has been adjusted to a concentration of 10 μg/ml using 0.1 M sodium carbonate buffer, pH 9.6. After allowing to stand at 4°C for 16 hours, the thus treated wells were washed three times with 10 mM phosphate buffer, pH 7.4, containing 0.05% Tween-20 (Bio-Rad Laboratories, Inc.) (to be referred to as "PBS- Tween" hereinafter). Each of the thus treated wells was charged with 300 μl of Block Ace (Dainippon Pharmaceutical Co., Ltd.) solution which has been diluted four times with water, incubated at 37°C for 1 hour to block un-adsorbed portion

and then washed three times with PBS-Tween. After adding 100 µl of the lysate and incubating at 37°C for 1.5 hours, each well was washed three times with PBS-Tween, charged with 100 µl of 10 µg/ml biotin-treated anti-human urine thrombomodulin antibody solution and then incubated at 37°C for 1 hour. After washing three times with PBS-Tween, 100 µl of a horse radish peroxidase-labeled streptoavidin (Zymed Laboratories, Inc.) solution was added and then incubated at 37°C for 1 hour. After washing three times with PBS-Tween, each well was washed once with citrate-phosphate buffer, pH 4.0, and then charged with 200 µl of a color-developing agent (ABTS: (2,2'-azinobis(3-ethylbenzthiazoline sulfonic acid)diammonium salt) which has been dissolved to a concentration of 1 mg/ml in citrate - phosphate buffer, pH 4.0, containing 0.003% hydrogen peroxide. The coloring reaction was continued until sufficient absorbance was obtained and then stopped by adding 50 µl of 21 mg/ml hydrogen fluoride solution to each well. Thereafter, absorbance at a wave length of 405 nm was measured using a microtiter plate reader.

As the results, color development was observed in the lysate of the strain containing pM450-TM-Ala or pM450-TM-Val, while no color development was observed in a lysate of *E. coli* strain HB101 containing plasmid pM450, which has been obtained in the same culture and preparation procedures.

Example 6

Construction and expression of deletion mutant

(1) Construction of deletion mutant expression vector

A vector for use in the expression of DEL 10 was constructed in the following manner. Oligonucleotides (D5-16U and D5-24L) comprising 10 mer and 24 mer as shown in Fig. 12(a) were prepared using a DNA synthesizer (already mentioned), purified by OPC column (already mentioned) and then annealed in the usual way to obtain a DNA fragment having cohesive end of *Kpn*I and *Eco*RI. This fragment was ligated in the usual way with a DNA fragment of about 4.5 kb prepared from a *Kpn*I/*Eco*RI digest of the pUC-TM obtained in Example 2-(1) to construct plasmid pUC-DEL10 which encodes human thrombomodulin signal peptide and DEL 10 supplemented with a terminal codon to its 3'-end. The thus constructed plasmid was digested with *Eco*RI/*Mlu*I, the digests were subjected to agarose gel electrophoresis in the usual way to isolate a DNA fragment of about 650 bp and then the thus isolated fragment was ligated in the usual way with a DNA fragment of about 4.5 kb prepared from a *Eco*RI/*Mlu*I digest of the pCDSR-α-TM obtained in Example 2-(2) to construct plasmid pCDSR-α-DEL10 (Fig. 13(a) - Fig. 13(b)).

On the other hand, a vector for use in the expression of a mutated recombinant human urine thrombomodulin in which C-terminal 49 amino acids are deleted from the human urine thrombomodulin (to be referred to as "DEL 49" hereinafter) was constructed in the following manner.

Oligonucleotides (D10 - 14U and D10 - 14L) each comprising 14 mer as shown in Fig. 12(b) were prepared using a DNA synthesizer (already mentioned), purified using OPC column (already mentioned) and then annealed in the usual way to obtain a DNA fragment having cohesive end of *Nhe*I and *Eco*RI. This fragment was ligated in the usual way with a DNA fragment of about 5 kb prepared from a *Eco*RI/*Nhe*I digest of the pCDSR α-TM obtained in Example 1-(2) to construct plasmid pCDSR α-DEL49 which contains the cDNA of interest. (Fig. 13(a) - Fig. 13(b))

(2) Expression in animal cells

The pCDSR α-TM prepared in Example 2-(2) and pCDSR α-DEL10 and pCDSR α-DEL49 prepared in Example 6-(1) were introduced into COS I cells to express ruTM-Ala, DEL 10 and DEL 49, respectively. That is, a 0.5 µg of pCDSR α-TM, pCDSR α-DEL10 or pCDSR α-DEL49 was dissolved in 5 µl of TE, and the resulting solution was mixed with 700 µl of D-ME medium containing 0.2 mg/ml of DEAE-dextran and 50 mM Tris- HCl (pH 7.4) to prepare a solution of DNA- DEAE-dextran mixture. The thus prepared DNA-DEAE-dextran mixture solution was added dropwise to COS I cells which have been cultured to a semi-confluent state in a culture dish of 35 mm in diameter, and the thus treated cells were cultured at 37°C for 4 hours in the presence of 5% CO_2-95% air. After removing the DNA- DEAE-dextran mixture solution, D-ME medium containing 1% fetal bovine serum (already mentioned) was added to the culture dish. After culturing at 37°C for 48 to 96 hours in the presence of 5% CO_2 - 95% air, the resulting culture supernatant was recovered and protein C activating ability of the supernatant was measured in accordance with the procedure of Experimental Example 2. As the results, the biological activity was found in ruTM-Ala and DEL 10, though not sufficiently enough in DEL 49. The results are shown in Table 4.

Table 4

	Activity *1
ruTM-Ala	3.8
DEL 10	4.1
Human placenta thrombomodulin	1.0

*1: Relative activity when the activity of human placenta thrombomodulin is defined as 1.

The following describes examples of pharmaceutical preparations containing the polypeptide of the present invention.

Example 7

ruTM-Ala	20.0 mg
Purified gelatin	50.0 mg
Sodium phosphate	34.8 mg
Sodium chloride	81.8 mg
Mannitol	25.0 mg

After dissolving the above components in 10 ml of distilled water for injection use, the resulting solution was sterilized by filtration, dispensed in 1.0 ml aliquots into sterile vials and then freeze-dried to prepare injections.

Example 8

ruTM-Ala	40.0 mg
Albumin	20.0 mg
Sodium phosphate	34.8 mg
Sodium chloride	81.8 mg
Mannitol	25.0 mg

After weighing each of the above components, a freeze-dried pharmaceutical preparation was prepared in the same manner as in Example 7.

Example 9

DEL 10	20.0 mg
Purified gelatin	50.0 mg
Sodium phosphate	34.8 mg
Sodium chloride	81.8 mg
Mannitol	25.0 mg

After weighing each of the above components, a freeze-dried pharmaceutical preparation was prepared in the same manner as in Example 7.

(Reference Example)

Example of the preparation of human placenta thrombomodulin

Thrombomodulin was purified from human placenta in accordance with the procedure disclosed in Japanese Patent Application Kokai No. 60-199819. That is, 12 kg of human placental samples (30 placentae) were washed with 0.02 M Tris-HCl buffer, pH 7.5, containing 0.25 M sucrose and 1 mM benzamidine and then homogenized using a meat grinder. After subjecting the thus homogenized suspension to centrifugation at 3,000 r.p.m. for 40 minutes, the resulting precipitate was suspended in the aforementioned buffer solution, stirred for 10 minutes and then centrifuged to obtain a precipitate. The above step was repeated three times using 20 liters of the buffer solution per one cycle, and the finally obtained precipitate was extracted with 60 liters of 0.02 M Tris-HCl buffer, pH 7.5, containing 0.25 M sucrose, 1 mM benzamidine hydrochloride and 0.5% (v/v) Triton X-100 (Sigma Chemical Co.). The amount of total protein in the thus extracted solution was found to be 46.7 g (determined by Lowry's method, the same shall apply hereinafter). The 60 liter crude extract was applied to DIP-thrombin-agarose column (4 ϕ x 16 cm) which has been equilibrated in advance with 0.02 M Tris-HCl buffer, pH 7.5, containing 0.1 M NaCl, 0.5 mM $CaCl_2$, 0.1 mM benzamidine hydrochloride and 0.5% (v/v) Triton X-100, and then the thus protein-adsorbed column was washed with 2 liters of the same buffer solution used for the equilibration. Next, elution was carried out using 0.02 M Tris-HCl buffer, pH 7.5, containing 1 M NaCl, 0.1 mM EDTA, 1 mM benzamidine hydrochloride and 0.5% (v/v) Triton X-100. In this way, 650 ml of eluate containing 1.7 g of protein was obtained. The eluate was subjected to desaltation and concentration using an ultrafiltration apparatus (Millipore Corp., nominal cutoff molecular weight of 30,000) and then adsorbed to the DIP-thrombin-agarose column which has been conditioned in the same manner as described above. Next, after washing with 150 ml of 0.02 M Tris-HCl buffer, pH 7.5, containing 0.4 M NaCl, 0.5 mM CaCl2, 0.1 mM benzamidine hydrochloride and 0.5% (v/v) Triton X-100, elution was carried out by means of density gradient using 0.02 M Tris-HCl buffer, pH 7.5, containing 0.1 mM EDTA, 1 mM benzamidine hydrochloride, 0.5% (v/v) Triton X-100 and NaCl (0.4 - 1 M). When the eluate was collected in 30 ml fractions, a total of 1290 ml fractions of interest containing 68 mg of protein was obtained. The eluate was subjected to desaltation and concentration using an ultrafiltration apparatus (Millipore Corp., nominal cutoff molecular weight of 30,000) and then to gel filtration to collect a fraction of interest using S-300 (Pharmacia) column (2.6 ϕ x 90 cm) which has been conditioned in advance with 0.01 M Tris-HCl buffer, pH 7.0, containing 0.05% Triton X-100 and 0.14 M NaCl. The thus obtained human placenta thrombomodulin preparation contained 3.1 mg of protein.

Brief Description of the Drawings

Fig. 1 is a graph showing oligonucleotide sequence of a probe to be used in the present invention.

Fig. 2 is a restriction map of a 2.5 kb cDNA fragment containing a DNA fragment which encodes the polypeptide of the present invention.

Fig. 3(a) to Fig. 3(m) include a graph showing oligonucleotide sequence of a 2.5 kb cDNA fragment containing a DNA fragment which encodes the polypeptide of the present invention and deduced amino acid sequence of the polypeptide.

Fig. 4(a) and Fig. 4(b) include a graph showing a procedure for the construction of expression plasmids pKCR-TM-Ala and pKCR-TM-Val of the present invention for use in mammalian cells.

Fig. 5(a) and Fig. 5(b) are graphs showing oligonucleotides used for the construction of the plasmids of the present invention.

Fig. 6(a) and Fig. 6(b) include a graph showing a procedure for the construction of expression plasmids pM450 -TM-Ala and pM450-TM-Val of the present invention for use in *E. coli.*

Fig. 7 is a graph showing an oligonucleotide used for the construction of the plasmid of the present invention.

Fig. 8 is a graph showing a procedure for the construction of plasmid pUC-TM containing a DNA fragment which encodes the polypeptide of the present invention.

Fig. 9(a) and Fig. 9(b) include a graph showing oligonucleotide sequence of a DNA fragment which encodes the polypeptide ruTM-Ala of the present invention.

Fig. 10(a) and Fig. 10(b) include a graph showing a procedure for the construction of expression plasmid LK-444-TM-DHFR of the present invention for use in mammalian cells.

Fig. 11(a) and Fig. 11(b) include a graph showing a procedure for the construction of expression plasmid pCDSR α-TM-DHFR of the present invention for use in mammalian cells.

Fig. 12 is a graph showing oligonucleotides used for the construction of deletion mutant expression plasmids pCDSR α-DEL10 and pCDSR α-DEL49 of the present invention.

Fig. 13(a) and Fig. 13(b) include a graph showing a procedure for the construction of expression plasmids pCDSR α-DEL10 and pCDSR α-DEL49 of the present invention for use in mammalian cells.

Industrial Applicability

The polypeptide of the present invention imparts an effect of inhibiting both blood coagulation and platelet aggregation because of its function to bind to thrombin and inactivate the activity thereof and, at the same time, exhibits anticoagulant and thrombolytic activities by activating protein C. Because of such effects, it is possible to use the polypeptide for the treatment of a broad range of hypercoagulability-related diseases, based on its thrombus formation inhibiting activity, thrombolytic activity, anti-DIC activity and the like. Especially, reduction of side effects can be expected because of its excellent function to activate protein C.

In addition, the polypeptide of the present invention has been produced for the first time by means of genetic engineering techniques. In consequence, when it is applied to a pharmaceutical drug as an agent for the treatment or prevention of hypercoagulability-related diseases such as thrombosis, DIC and the like, more stronger effect than the prior art counterpart, or similar effect with smaller dose, can be expected, thus rendering possible economical use of the drug with less danger of generating side effects. Also, it is possible to find an entirely new effect such as treatment of a disease which is difficult to cure in the present situation.

Also, the polypeptide of the present invention can be used more safely as a pharmaceutical drug, because it is not necessary to use a surface active agent which is essential for the solubilization of prior art human thrombomodulin extracted from tissues of placenta, the lungs and the like.

In addition to its application to pharmaceutical drugs as described above, the polypeptide of the present invention can also be used for the purpose of preventing blood coagulation, by binding and adsorbing it to the surface of an artificial blood vessel, an artificial organ, a catheter or the like making use of a cross-linking agent or the like.

Claims

1. A polypeptide prepared by means of genetic recombinantion techniques which comprises an amino acid sequence represented by the following formula:

X_1 Glu Pro Gln Pro Gly Gly Ser Gln Cys Val Glu
5 10
His Asp Cys Phe Ala Leu Tyr Pro Gly Pro Ala Thr
15 20 25
Phe Leu Asn Ala Ser Gln Ile Cys Asp Gly Leu Arg
30 35
Gly His Leu Met Thr Val Arg Ser Ser Val Ala Ala
40 45 50
Asp Val Ile Ser Leu Leu Leu Asn Gly Asp Gly Gly
55 60
Val Gly Arg Arg Arg Leu Trp Ile Gly Leu Gln Leu
65 70
Pro Pro Gly Cys Gly Asp Pro Lys Arg Leu Gly Pro
75 80 85
Leu Arg Gly Phe Gln Trp Val Thr Gly Asp Asn Asn
90 95
Thr Ser Tyr Ser Arg Trp Ala Arg Leu Asp Leu Asn
100 105 110
Gly Ala Pro Leu Cys Gly Pro Leu Cys Val Ala Val
115 120
Ser Ala Ala Glu Ala Thr Val Pro Ser Glu Pro Ile
125 130
Trp Glu Glu Gln Gln Cys Glu Val Lys Ala Asp Gly
135 140 145

```
Phe Leu Cys Glu Phe His Phe Pro Ala Thr Cys Arg
                150                 155
Pro Leu Ala Val Glu Pro Gly Ala Ala Ala Ala Ala
    160                 165                 170
Val Ser Ile Thr Tyr Gly Thr Pro Phe Ala Ala Arg
                175                 180
Gly Ala Asp Phe Gln Ala Leu Pro Val Gly Ser Ser
        185                 190
Ala Ala Val Ala Pro Leu Gly Leu Gln Leu Met Cys
195                 200                 205
Thr Ala Pro Pro Gly Ala Val Gln Gly His Trp Ala
                210                 215
Arg Glu Ala Pro Gly Ala Trp Asp Cys Ser Val Glu
    220                 225                 230
Asn Gly Gly Cys Glu His Ala Cys Asn Ala Ile Pro
                235                 240
Gly Ala Pro Arg Cys Gln Cys Pro Ala Gly Ala Ala
        245                 250
Leu Gln Ala Asp Gly Arg Ser Cys Thr Ala Ser Ala
255                 260                 265
Thr Gln Ser Cys Asn Asp Leu Cys Glu His Phe Cys
                270                 275
Val Pro Asn Pro Asp Gln Pro Gly Ser Tyr Ser Cys
    280                 285                 290
Met Cys Glu Thr Gly Tyr Arg Leu Ala Ala Asp Gln
                295                 300
His Arg Cys Glu Asp Val Asp Asp Cys Ile Leu Glu
        305                 310
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Pro Ser Pro Cys Pro Gln Arg Cys Val Asn Thr Gln
315                 320                 325
Gly Gly Phe Glu Cys His Cys Tyr Pro Asn Tyr Asp
            330                 335
Leu Val Asp Gly Glu Cys Val Glu Pro Val Asp Pro
    340                 345                 350
Cys Phe Arg Ala Asn Cys Glu Tyr Gln Cys Gln Pro
                355                 360
Leu Asn Gln Thr Ser Tyr Leu Cys Val Cys Ala Glu
        365                 370
Gly Phe Ala Pro Ile Pro His Glu Pro His Arg Cys
375                 380                 385
Gln Met Phe Cys Asn Gln Thr Ala Cys Pro Ala Asp
            390                 395
Cys Asp Pro Asn Thr Gln Ala Ser Cys Glu Cys Pro
    400                 405                 410
Glu Gly Tyr Ile Leu Asp Asp Gly Phe Ile Cys Thr
                415                 420
Asp Ile Asp Glu Cys Glu Asn Gly Gly Phe Cys Ser
        425                 430
Gly Val Cys His Asn Leu Pro Gly Thr Phe Glu Cys
435                 440                 445
```

Y_1

[in this formula, X_1 is a sequence represented by the following formula:

```
Met Leu Gly Val Leu Val Leu Gly Ala Leu Ala Leu
            -15                 -10
Ala Gly Leu Gly Phe Pro Ala Pro Ala
     -5             -1   1
```

or its variation in which optional number or entire amino acids are deleted starting from its N-terminus, and Y_1 is a sequence represented by the following formula:

```
Ile Cys Gly Pro Asp Ser Ala Leu  Z  Arg His
                                 450      455
```

[in this instance, Z is Val or Ala]
or its variation in which optional number or entire amino acids are deleted starting from its C-terminus], wherein, optionally, at least one amino acid of said sequence may have a sugar chain.

2. The polypeptide according to claim 1 wherein, optionally, at least one amino acid of said amino acid sequence may have a sugar chain, X_1 is an amino acid sequence represented by the following formula:

```
Ala Pro Ala
  1
```

and Y_1 is an amino acid sequence represented by the following formula:

```
Ile Cys Gly Pro Asp Ser Ala Leu  Z  Arg
                                 450      455
```

[in this instance, Z is Val or Ala].

3. The polypeptide according to claim 1 wherein, optionally, at least one amino acid of said amino acid sequence may have a sugar chain, X_1 is an amino acid sequence represented by the following formula:

```
Ala Pro Ala
  1
```

and entire amino acids of Y_1 are deleted.

4. The polypeptide according to claims 1 to 3 wherein at least one amino acid of said amino acid sequence has a sugar chain.

5. The polypeptide according to claims 1 to 3 wherein none of amino acids of said sequence have sugar chain.

6. A DNA fragment which encodes the polypeptide of claims 1 to 5.

7. The DNA fragment according to claim 6 wherein said fragment comprises a nucleotide sequence represented by the following formula:

X_2 GAGCCGC AGCCGGGTGG CAGCCAGTGC GTCGAGCACG 100

ACTGCTTCGC GCTCTACCCG GGCCCCGCGA CCTTCCTCAA 140

TGCCAGTCAG ATCTGCGACG GACTGCGGGG CCACCTAATG 180

ACAGTGCGCT CCTCGGTGGC TGCCGATGTC ATTTCCTTGC 220

TACTGAACGG CGACGGCGGC GTTGGCCGCC GGCGCCTCTG 260

GATCGGCCTG CAGCTGCCAC CCGGCTGCGG CGACCCCAAG 300

CGCCTCGGGC CCCTGCGCGG CTTCCAGTGG GTTACGGGAG 340

ACAACAACAC CAGCTATAGC AGGTGGGCAC GGCTCGACCT 380

CAATGGGGCT CCCCTCTGCG GCCCGTTGTG CGTCGCTGTC 420

TCCGCTGCTG AGGCCACTGT GCCCAGCGAG CCGATCTGGG 460

AGGAGCAGCA GTGCGAAGTG AAGGCCGATG GCTTCCTCTG 500

CGAGTTCCAC TTCCCAGCCA CCTGCAGGCC ACTGGCTGTG 540

GAGCCCGGCG CCGCGGCTGC CGCCGTCTCG ATCACCTACG 580

GCACCCCGTT CGCGGCCCGC GGAGCGGACT TCCAGGCGCT 620

GCCGGTGGGC AGCTCCGCCG CGGTGGCTCC CCTCGGCTTA 660

CAGCTAATGT GCACCGCGCC GCCCGGAGCG GTCCAGGGGC 700

ACTGGGCCAG GGAGGCGCCG GGCGCTTGGG ACTGCAGCGT 740

GGAGAACGGC GGCTGCGAGC ACGCGTGCAA TGCGATCCCT 780

GGGGCTCCCC GCTGCCAGTG CCCAGCCGGC GCCGCCCTGC 820

AGGCAGACGG GCGCTCCTGC ACCGCATCCG CGACGCAGTC 860

CTGCAACGAC CTCTGCGAGC ACTTCTGCGT TCCCAACCCC 900

GACCAGCCGG GCTCCTACTC GTGCATGTGC GAGACCGGCT 940

ACCGGCTGGC GGCCGACCAA CACCGGTGCG AGGACGTGGA 980

TGACTGCATA CTGGAGCCCA GTCCGTGTCC GCAGCGCTGT 1020

GTCAACACAC AGGGTGGCTT CGAGTGCCAC TGCTACCCTA 1060

ACTACGACCT GGTGGACGGC GAGTGTGTSG AGCCCGTGGA 1100

CCCGTGCTTC AGAGCCAACT GCGAGTACCA GTGCCAGCCC 1140

CTGAACCAAA CTAGCTACCT CTGCGTCTGC GCCGAGGGCT 1180

TCGCGCCCAT TCCCCACGAG CCGCACAGGT GCCAGATGTT 1220

TTGCAACCAG ACTGCCTGTC CAGCCGACTG CGACCCCAAC 1260

ACCCAGGCTA GCTGTGAGTG CCCTGAAGGC TACATCCTGG 1300

ACGACGGTTT CATCTGCACG GACATCGACG AGTGCGAAAA 1340

CGGCGGCTTC TGCTCCGGGG TGTGCCACAA CCTCCCCGGT 1380

ACCTTCGAGT GC Y_2 1392

[in this formula, S is G or C; X_2 is a sequence represented by the following formula:

ATGCTTGGGG TCCTGGTCCT TGGCGCGCTG GCCCTGGCCG 40

GCCTGGGGTT CCCCGCWCCC GCA 63

[provided that W is T or A]
or its variation in which optional number or entire nucleotides are deleted in triplets starting from its 5'-end; and Y_2 is a sequence represented by the following formula:

ATCTGCGGGC CCGACTCGGC CCTTGYCCGC CAC 1425

[provided that Y is T or C]
or its variation in which optional number or entire nucleotides are deleted in triplets starting from its 3'-end].

8. The DNA fragment according to claim 7 wherein X_2 is a nucleotide sequence represented by the following formula:

GCWCCCGCA 63

[in this formula, W is T or A]
and Y_2 is a nucleotide sequence represented by the following formula:

ATCTGCGGGC CCGACTCGGC CCTTGYCCGC 1422

[in this instance, Y is T or C]

9. The DNA fragment according to claim 7 wherein X_2 is a nucleotide sequence represented by the following formula:

GCWCCCGCA 63

[in this formula, W is T or A]
and entire nucleotides of Y_2 are deleted.

10. A process for producing the polypeptide of claims 1 to 5 which comprises performing at least one step selected from the following steps of:
a) preparing a DNA fragment containing a nucleotide sequence which encodes said polypeptide,
b) incorporating said DNA fragment into an expression vector to obtain a recombinant DNA fragment which contains said DNA fragment and is capable of undergoing replication,
c) transforming a host cell with said recombinant DNA fragment to isolate a transformant which can express said polypeptide, and
d) culturing said transformant to allow the transformant to produce said polypeptide and recovering said polypeptide from resulting cultured mixture.

11. The process for producing polypeptide according to claim 10 wherein said host cell is a eukaryotic cell.

12. The process for producing polypeptide according to claim 10 wherein said host cell is a prokaryotic cell.

13. An agent for use in the prevention and treatment of hypercoagulability related diseases which comprises the polypeptide of claim 1 as an active ingredient.

14. An agent for use in the prevention and treatment of hypercoagulability related diseases which comprises the polypeptide of claim 2 as an active ingredient.

15. An agent for use in the prevention and treatment of hypercoagulability related diseases which comprises the polypeptide of claim 3 as an active ingredient.

16. An agent for use in the prevention and treatment of hypercoagulability related diseases which comprises the polypeptide of claim 4 as an active ingredient.

17. An agent for use in the prevention and treatment of hypercoagulability related diseases which comprises the polypeptide of claim 5 as an active ingredient.

FIG. 1

G C A A A A C A A T	C A T G T T C	1 7
G C G A A G C A G T	C G T G C T C	1 7

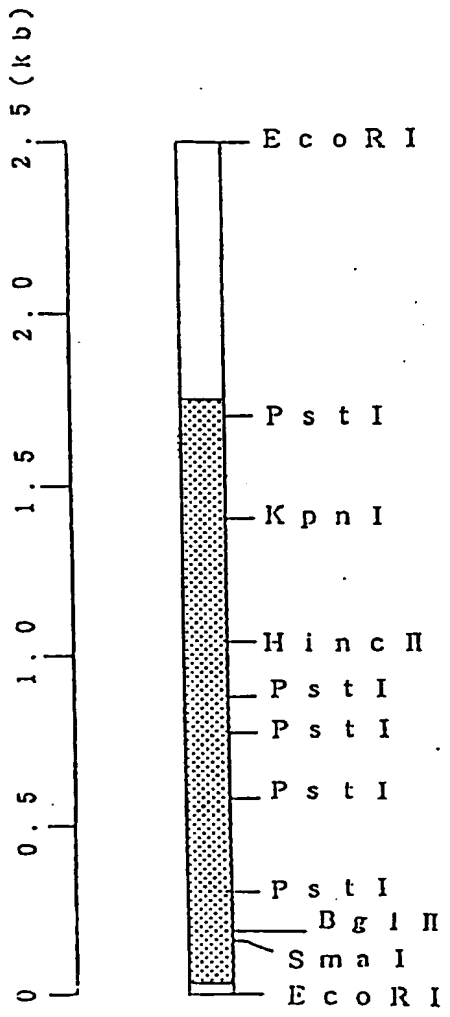

2.5(kb)
2.0
1.5
1.0
0.5
0
EcoRI
PstI
KpnI
HincII
PstI
PstI
PstI
PstI
BglII
SmaI
EcoRI

FIG. 2

FIG. 3(a)

```
GCTTTCCCCG GCGCCTGCAC GCGGCGCGCC TGGGTAAC ATG      41
                                           Met

CTT GGG GTC CTG GTC CTT GGC GCG CTG GCC CTG        74
Leu Gly Val Leu Val Leu Gly Ala Leu Ala Leu
        -15                 -10

GCC GGC CTG GGG TTC CCC GCA CCC GCA GAG CCG       107
Ala Gly Leu Gly Phe Pro Ala Pro Ala Glu Pro
     -5             -1  1                5

CAG CCG GGT GGC AGC CAG TGC GTC GAG CAC GAC       140
Gln Pro Gly Gly Ser Gln Cys Val Glu His Asp
                10                  15

TGC TTC GCG CTC TAC CCG GGC CCC GCG ACC TTC       173
Cys Phe Ala Leu Tyr Pro Gly Pro Ala Thr Phe
            20                  25
```

FIG. 3(b)

```
CTC AAT GCC AGT CAG ATC TGC GAC GGA CTG CGG     206
Leu Asn Ala Ser Gln Ile Cys Asp Gly Leu Arg
          30                  35

GGC CAC CTA ATG ACA GTG CGC TCC TCG GTG GCT     239
Gly His Leu Met Thr Val Arg Ser Ser Val Ala
      40                  45

GCC GAT GTC ATT TCC TTG CTA CTG AAC GGC GAC     272
Ala Asp Val Ile Ser Leu Leu Leu Asn Gly Asp
 50                  55                  60

GGC GGC GTT GGC CGC CGG CGC CTC TGG ATC GGC     305
Gly Gly Val Gly Arg Arg Arg Leu Trp Ile Gly
                 65                  70

CTG CAG CTG CCA CCC GGC TGC GGC GAC CCC AAG     338
Leu Gln Leu Pro Pro Gly Cys Gly Asp Pro Lys
               75                  80
```

FIG. 3(c)

```
CGC CTC GGG CCC CTG CGC GGC TTC CAG TGG GTT     371
Arg Leu Gly Pro Leu Arg Gly Phe Gln Trp Val
          85                  90

ACG GGA GAC AAC AAC ACC AGC TAT AGC AGG TGG     404
Thr Gly Asp Asn Asn Thr Ser Tyr Ser Arg Trp
     95                  100

GCA CGG CTC GAC CTC AAT GGG GCT CCC CTC TGC     437
Ala Arg Leu Asp Leu Asn Gly Ala Pro Leu Cys
105                 110                 115

GGC CCG TTG TGC GTC GCT GTC TCC GCT GCT GAG     470
Gly Pro Leu Cys Val Ala Val Ser Ala Ala Glu
                120                 125

GCC ACT GTG CCC AGC GAG CCG ATC TGG GAG GAG     503
Ala Thr Val Pro Ser Glu Pro Ile Trp Glu Glu
          130                 135
```

FIG. 3(d)

```
CAG CAG TGC GAA GTG AAG GCC GAT GGC TTC CTC      536
Gln Gln Cys Glu Val Lys Ala Asp Gly Phe Leu
        140                 145

TGC GAG TTC CAC TTC CCA GCC ACC TGC AGG CCA      569
Cys Glu Phe His Phe Pro Ala Thr Cys Arg Pro
    150                 155

CTG GCT GTG GAG CCC GGC GCC GCG GCT GCC GCC      602
Leu Ala Val Glu Pro Gly Ala Ala Ala Ala Ala
160                 165                 170

GTC TCG ATC ACC TAC GGC ACC CCG TTC GCG GCC      635
Val Ser Ile Thr Tyr Gly Thr Pro Phe Ala Ala
                175                 180

CGC GGA GCG GAC TTC CAG GCG CTG CCG GTG GGC      668
Arg Gly Ala Asp Phe Gln Ala Leu Pro Val Gly
            185                 190
```

FIG. 3(e)

```
AGC TCC GCC GCG GTG GCT CCC CTC GGC TTA CAG      701
Ser Ser Ala Ala Val Ala Pro Leu Gly Leu Gln
        195                 200

CTA ATG TGC ACC GCG CCG CCC GGA GCG GTC CAG      734
Leu Met Cys Thr Ala Pro Pro Gly Ala Val Gln
    205                 210

GGG CAC TGG GCC AGG GAG GCG CCG GGC GCT TGG      767
Gly His Trp Ala Arg Glu Ala Pro Gly Ala Trp
215                 220                 225

GAC TGC AGC GTG GAG AAC GGC GGC TGC GAG CAC      800
Asp Cys Ser Val Glu Asn Gly Gly Cys Glu His
                230                 235

GCG TGC AAT GCG ATC CCT GGG GCT CCC CGC TGC      833
Ala Cys Asn Ala Ile Pro Gly Ala Pro Arg Cys
            240                 245
```

FIG. 3(f)

```
CAG TGC CCA GCC GGC GCC GCC CTG CAG GCA GAC      866
Gln Cys Pro Ala Gly Ala Ala Leu Gln Ala Asp
        250                 255

GGG CGC TCC TGC ACC GCA TCC GCG ACG CAG TCC      899
Gly Arg Ser Cys Thr Ala Ser Ala Thr Gln Ser
    260                 265

TGC AAC GAC CTC TGC GAG CAC TTC TGC GTT CCC      932
Cys Asn Asp Leu Cys Glu His Phe Cys Val Pro
270                 275                 280

AAC CCC GAC CAG CCG GGC TCC TAC TCG TGC ATG      965
Asn Pro Asp Gln Pro Gly Ser Tyr Ser Cys Met
                285                 290

TGC GAG ACC GGC TAC CGG CTG GCG GCC GAC CAA      998
Cys Glu Thr Gly Tyr Arg Leu Ala Ala Asp Gln
            295                 300
```

FIG. 3(g)

```
CAC CGG TGC GAG GAC GTG GAT GAC TGC ATA CTG    1031
His Arg Cys Glu Asp Val Asp Asp Cys Ile Leu
    305                 310

GAG CCC AGT CCG TGT CCG CAG CGC TGT GTC AAC    1064
Glu Pro Ser Pro Cys Pro Gln Arg Cys Val Asn
315                 320

ACA CAG GGT GGC TTC GAG TGC CAC TGC TAC CCT    1097
Thr Gln Gly Gly Phe Glu Cys His Cys Tyr Pro
325                 330                 335

AAC TAC GAC CTG GTG GAC GGC GAG TGT GTG GAG    1130
Asn Tyr Asp Leu Val Asp Gly Glu Cys Val Glu
                340                 345

CCC GTG GAC CCG TGC TTC AGA GCC AAC TGC GAG    1163
Pro Val Asp Pro Cys Phe Arg Ala Asn Cys Glu
            350                 355
```

FIG. 3(h)

```
TAC CAG TGC CAG CCC CTG AAC CAA ACT AGC TAC   1196
Tyr Gln Cys Gln Pro Leu Asn Gln Thr Ser Tyr
        360                 365

CTC TGC GTC TGC GCC GAG GGC TTC GCG CCC ATT   1229
Leu Cys Val Cys Ala Glu Gly Phe Ala Pro Ile
    370                 375

CCC CAC GAG CCG CAC AGG TGC CAG ATG TTT TGC   1262
Pro His Glu Pro His Arg Cys Gln Met Phe Cys
380                 385                 390

AAC CAG ACT GCC TGT CCA GCC GAC TGC GAC CCC   1295
Asn Gln Thr Ala Cys Pro Ala Asp Cys Asp Pro
                395                 400

AAC ACC CAG GCT AGC TGT GAG TGC CCT GAA GGC   1328
Asn Thr Gln Ala Ser Cys Glu Cys Pro Glu Gly
            405                 410
```

FIG. 3(i)

```
TAC ATC CTG GAC GAC GGT TTC ATC TGC ACG GAC   1361
Tyr Ile Leu Asp Asp Gly Phe Ile Cys Thr Asp
        415                 420

ATC GAC GAG TGC GAA AAC GGC GGC TTC TGC TCC   1394
Ile Asp Glu Cys Glu Asn Gly Gly Phe Cys Ser
    425                 430

GGG GTG TGC CAC AAC CTC CCC GGT ACC TTC GAG   1427
Gly Val Cys His Asn Leu Pro Gly Thr Phe Glu
435                 440                 445

TGC ATC TGC GGG CCC GAC TCG GCC CTT GCC CGC   1460
Cys Ile Cys Gly Pro Asp Ser Ala Leu Ala Arg
                450                 455

CAC ATT GGC ACC GAC TGT GAC TCC GGC AAG GTG   1493
His Ile Gly Thr Asp Cys Asp Ser Gly Lys Val
            460                 465
```

FIG. 3(j)

```
GAC GGT GGC GAC AGC GGC TCT GGC GAG CCC CCG    1526
Asp Gly Gly Asp Ser Gly Ser Gly Glu Pro Pro
        470                 475

CCC AGC CCG ACG CCC GGC TCC ACC TTG ACT CCT    1559
Pro Ser Pro Thr Pro Gly Ser Thr Leu Thr Pro
    480                 485

CCG GCC GTG GGG CTC GTG CAT TCG GGC TTG CTC    1592
Pro Ala Val Gly Leu Val His Ser Gly Leu Leu
490                 495                 500

ATA GGC ATC TCC ATC GCG AGC CTG TGC CTG GTG    1625
Ile Gly Ile Ser Ile Ala Ser Leu Cys Leu Val
                505                 510

GTG GCG CTT TTG GCG CTC CTC TGC CAC CTG CGC    1658
Val Ala Leu Leu Ala Leu Leu Cys His Leu Arg
            515                 520
```

FIG. 3(k)

```
AAG AAG CAG GGC GCC GCC AGG GCC AAG ATG GAG    1691
Lys Lys Gln Gly Ala Ala Arg Ala Lys Met Glu
        525                 530

TAC AAG TGC GCG GCC CCT TCC AAG GAG GTA GTG    1724
Tyr Lys Cys Ala Ala Pro Ser Lys Glu Val Val
    535                 540

CTG CAG CAC GTG CGG ACC GAG CGG ACG CCG CAG    1757
Leu Gln His Val Arg Thr Glu Arg Thr Pro Gln
545                 550                 555

AGA CTC TGA GCGG CCTCCGTCCA GGAGCCTGGC         1790
Arg Leu ***

TCCGTCCAGG AGCCTGTGCC TCCTCACCCC CAGCTTTGCT    1830

ACCAAAGCAC CTTAGCTGGC ATTACAGCTG GAGAAGACCC    1870

TCCCCGCACC CCCCAAGCTG TTTTCTTCTA TTCCATGGCT    1910
```

FIG. 3(1)

```
AACTGGCGAG GGGGTGATTA GAGGGAGGAG AATGAGCCTC   1950

GGCCTCTTCC GTGACGTCAC TGGACCACTG GGCAATGATG   1990

GCAATTTTGT AACGAAGACA CAGACTGCGA TTTGTCCCAG   2030

GTCCTCACTA CCGGGCGCAG GAGGGTGAGC GTTATTGGTC   2070

GGCAGCCTTC TGGGCAGACC TTGACCTCGT GGGCTAGGGA   2110

TGACTAAAAT ATTTATTTTT TTTAAGTATT TAGGTTTTTG   2150

TTTGTTTCCT TTGTTCTTAC CTGTATGTCT CCAGTATCCA   2190

CTTTGCACAG CTCTCCGGTC TCTCTCTCTC TACAAACTCC   2230

CACTTGTCAT GTGACAGGTA AACTATCTTG GTGAATTTTT   2270

TTTTCCTAGC CCTCTCACAT TTATGAAGCA AGCCCCACTT   2310

ATTCCCCATT CTTCCTAGTT TTCTCCTCCC AGGAACTGGG   2350
```

FIG. 3(m)

```
CCAACTCACC TGAGTCACCC TACCTGTGCC TGACCCTACT    2390

TCTTTTGCTC TTAGCTGTCT GCTCAGACAG AACCCCTACA    2430

TGAAACAGAA ACAAAAACAC TAAAAATAAA AAT           2463
```

FIG. 4(a)

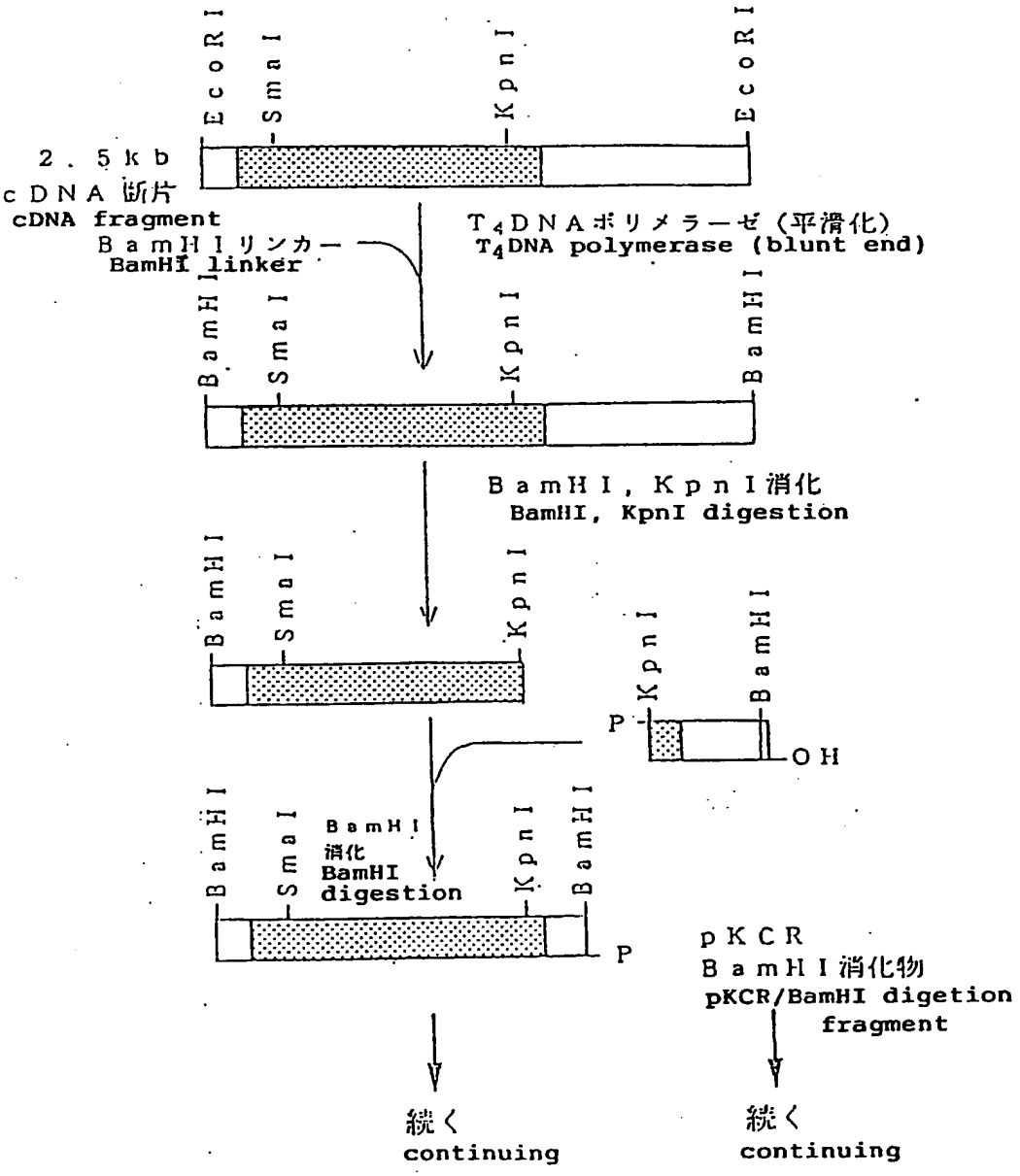

EcoRI
SmaI
KpnI
EcoRI
2.5kb
cDNA 断片
cDNA fragment
BamHIリンカー
BamHI linker
T4DNAポリメラーゼ（平滑化）
T4DNA polymerase (blunt end)
BamHI
SmaI
KpnI
BamHI
BamHI, KpnI消化
BamHI, KpnI digestion
BamHI
SmaI
KpnI
P
KpnI
BamHI
OH
BamHI
SmaI
BamHI
消化
BamHI
digestion
KpnI
BamHI
P
pKCR
BamHI消化物
pKCR/BamHI digetion
fragment
続く
continuing
続く
continuing

FIG. 4(b)

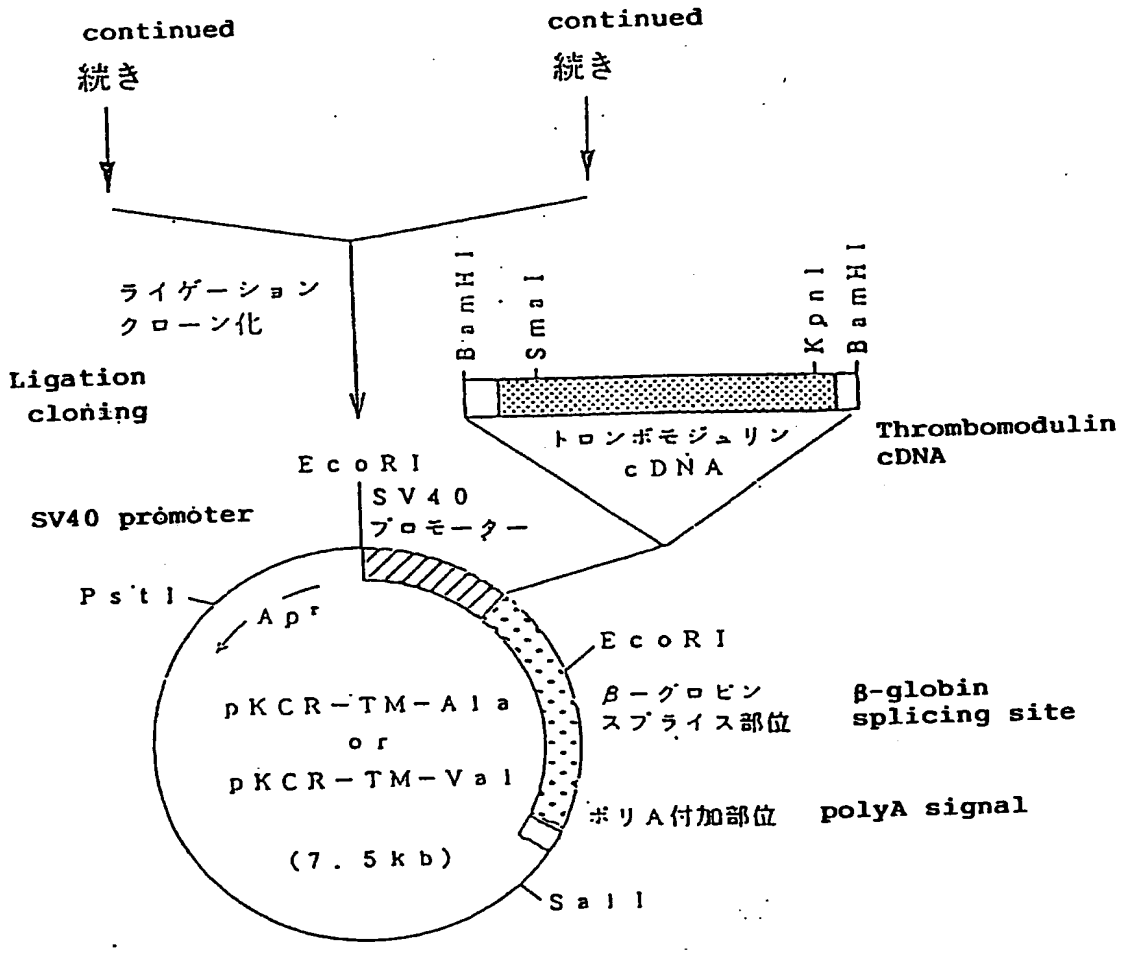

continued
続き
continued
続き
ライゲーション
クローン化
Ligation
cloning
BamHI
SmaI
KpnI
BamHI
トロンボモジュリン
cDNA
Thrombomodulin
cDNA
EcoRI
SV40
プロモーター
SV40 promoter
PstI
Apr
EcoRI
pKCR-TM-Ala
or
pKCR-TM-Val
(7.5kb)
βーグロビン
スプライス部位
β-globin
splicing site
ポリA付加部位
polyA signal
SalI

FIG. 5(a)

pKCR－TM－Ala作製用
Oligonucleotides for pKCR-TM-Ala construction

49 mer	CTTCGAGTGC ATCTGCGGGC CCGACTCGGC	30
	CCTTGCCCGC TAGGATCCC	49
53 mer	GGGATCCTAG CGGGCAAGGG CCGAGTCGGG	30
	CCCGCAGATG CACTCGAAGG TAC	53

pKCR－TM－Val作製用
Oligonucleotides for pKCR-TM-Val construction

49 mer	CTTCGAGTGC ATCTGCGGGC CCGACTCGGC	30
	CCTTGTCCGC TAGGATCCC	49
53 mer	GGGATCCTAG CGGACAAGGG CCGAGTCGGG	30
	CCCGCAGATG CACTCGAAGG TAC	53

FIG. 5(b)

pM450-TM-Ala およびpM450-TM-Val作製用
Oligonucleotides for pM450-TM-Ala and pM450-TM-Val constructions

69 mer	TATGGCACCA GCAGAACCAC AACCAGGTGG	30
	AAGTCAATGT GTAGAACATG ATTGTTTTGC	60
	ACTATATCC	69
67 mer	GGATATAGTG CAAAACAATC ATGTTCTACA	30
	CATTGACTTC CACCTGGTTG TGGTTCTGCT	60
	GGTGCCA	67

FIG. 6(a)

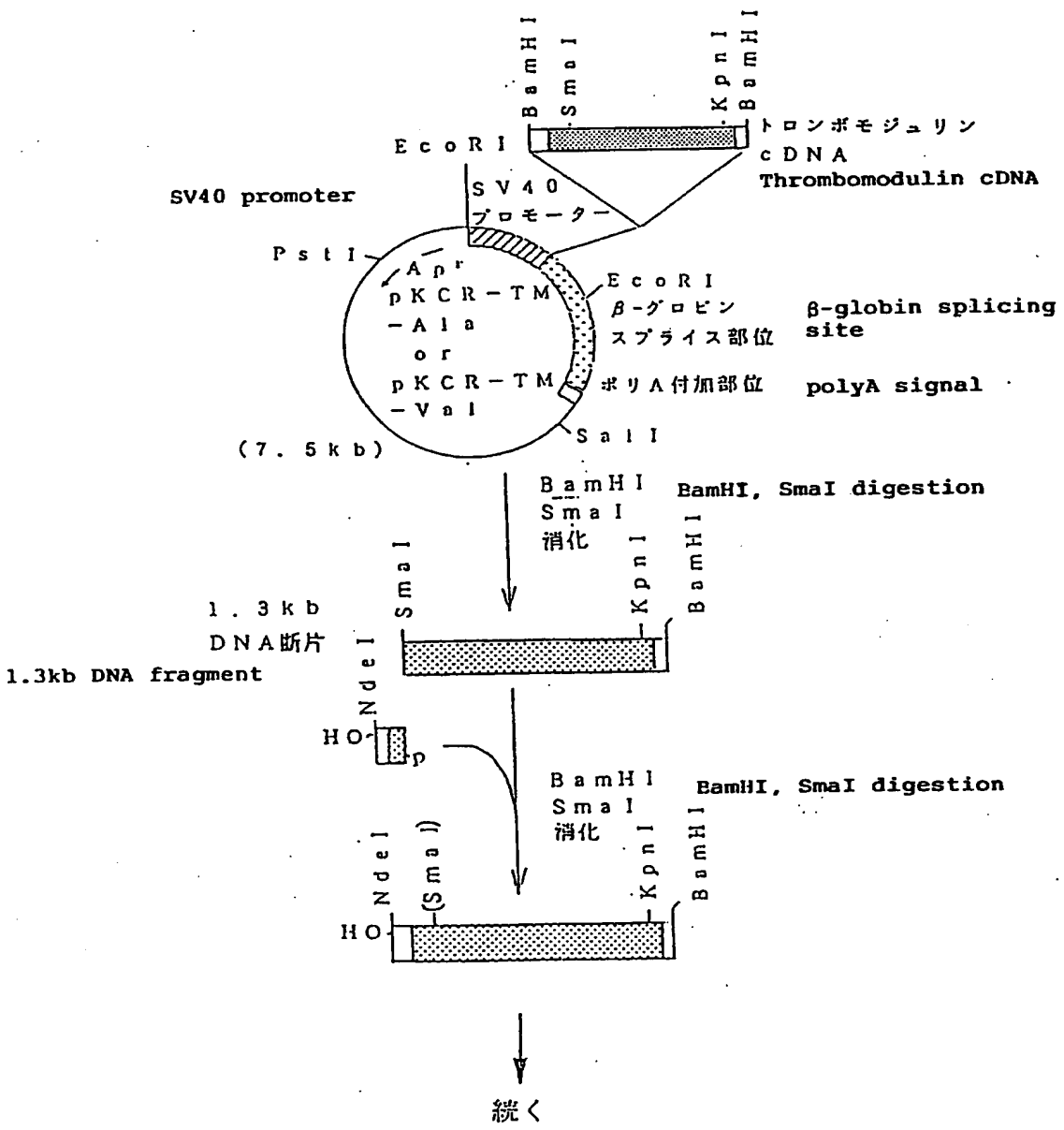

BamHI
SmaI
KpnI
BamHI
トロンボモジュリン
cDNA
Thrombomodulin cDNA
EcoRI
SV40 promoter
SV40
プロモーター
PstI
Apr
pKCR-TM
-Ala
or
pKCR-TM
-Val
EcoRI
β-グロビン
スプライス部位
β-globin splicing site
ポリA付加部位
polyA signal
SalI
(7.5kb)
BamHI
SmaI
消化
BamHI, SmaI digestion
1.3kb
DNA断片
1.3kb DNA fragment
SmaI
KpnI
BamHI
NdeI
HO
p
BamHI
SmaI
消化
BamHI, SmaI digestion
NdeI
(SmaI)
KpnI
BamHI
HO
続く
continuing

FIG. 6(b)

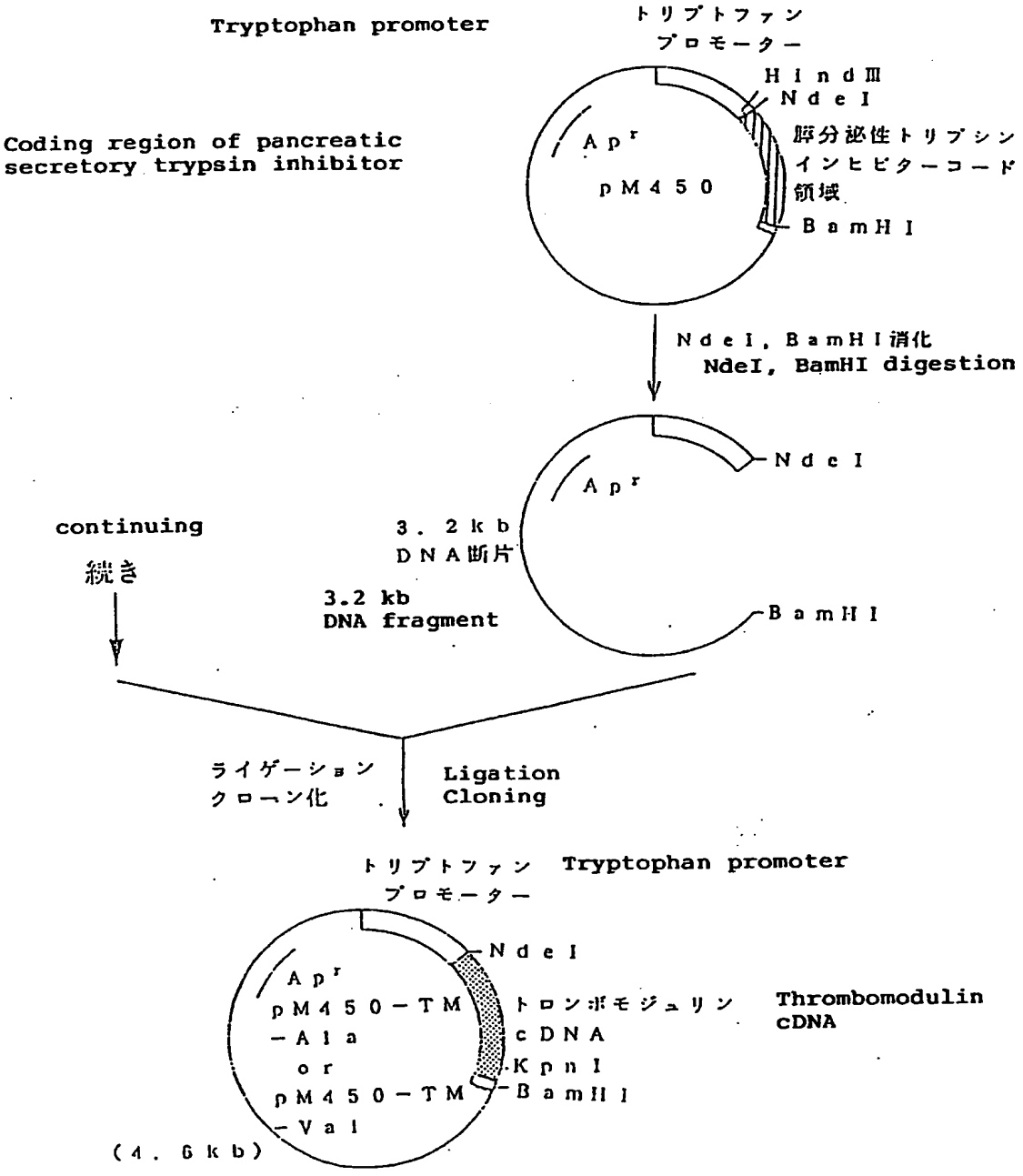

Tryptophan promoter
トリプトファン
プロモーター
HindⅢ
NdeI
Coding region of pancreatic secretory trypsin inhibitor
Apr
pM450
膵分泌性トリプシン
インヒビターコード
領域
BamHI
NdeI, BamHI消化
NdeI, BamHI digestion
NdeI
Apr
continuing
続き
3．2kb
DNA断片
3.2 kb
DNA fragment
BamHI
ライゲーション
クローン化
Ligation
Cloning
トリプトファン
プロモーター
Tryptophan promoter
NdeI
Apr
pM450－TM
－Ala
or
pM450－TM
－Val
トロンボモジュリン
cDNA
KpnI
BamHI
Thrombomodulin
cDNA
(4．6kb)

FIG. 7

S1 TTGTCGACAT GCTTGGGGTC CTGGTCCTT 29

Sal I

S2 ATAAGCTTCC GCTGCTGAGG CCACTGTGC 29

Hind III

S3 TTCTGCAGCT CGAGCCCGTG GACCCGTGCT TC 32

Pst I Xho I

A1 TTGGATCCCA CAGTGGCCTC AGCAGCGGA 29

BamH I

A2 ATGTCGACAC ACTCGCCGTC CACCAGGTC 29

Sal I

A3 GCGAATTCGG ATCCTCAGC GGGCAAGGGCC GAGTCGGG 38

EcoR I BamH I

FIG. 8

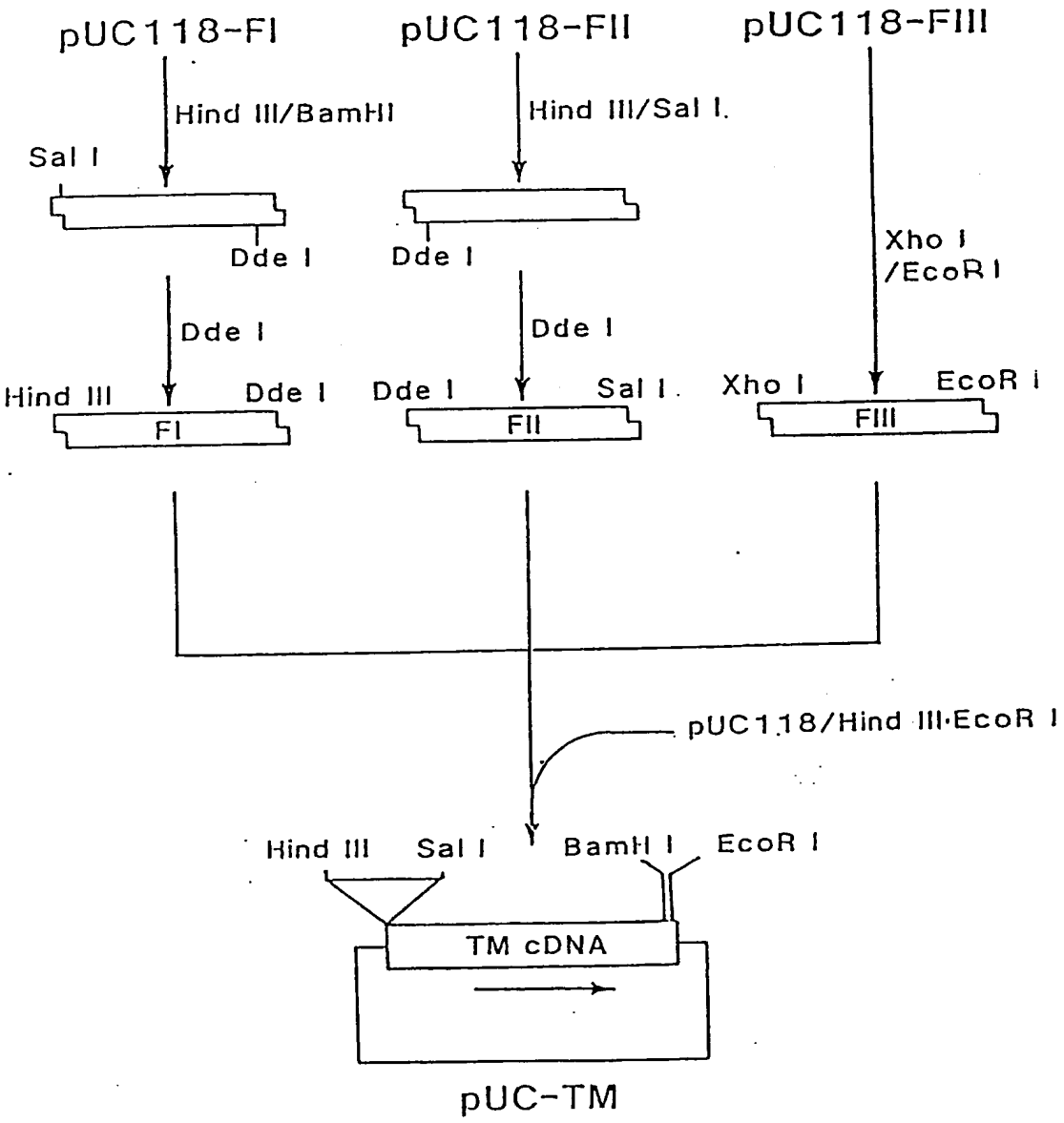

pUC118-FI
pUC118-FII
pUC118-FIII
Hind III/BamHI
Hind III/Sal I.
Sal I
Dde I
Dde I
Xho I
/EcoR I
Dde I
Dde I
Hind III
Dde I
Dde I
Sal I.
Xho I
EcoR I
FI
FII
FIII
pUC118/Hind III·EcoR I
Hind III
Sal I
BamH I
EcoR I
TM cDNA
pUC-TM

FIG. 9(a)

```
ATGCTTGGGG TCCTGGTCCT TGGCGCGCTG GCCCTGGCCG      40
GCCTGGGGTT CCCCGCTCCC GCAGAGCCGC AGCCGGGTGG      80
CAGCCAGTGC GTCGAGCACG ACTGCTTCGC GCTCTACCCG     120
GGCCCCGCGA CCTTCCTCAA TGCCAGTCAG ATCTGCGACG     160
GACTGCGGGG CCACCTAATG ACAGTGCGCT CCTCGGTGGC     200
TGCCGATGTC ATTTCCTTGC TACTGAACGG CGACGGCGGC     240
GTTGGCCGCC GGCGCCTCTG GATCGGCCTG CAGCTGCCAC     280
CCGGCTGCGG CGACCCCAAG CGCCTCGGGC CCCTGCGCGG     320
CTTCCAGTGG GTTACGGGAG ACAACAACAC CAGCTATAGC     360
AGGTGGGCAC GGCTCGACCT CAATGGGGCT CCCCTCTGCG     400
GCCCGTTGTG CGTCGCTGTC TCCGCTGCTG AGGCCACTGT     440
GCCCAGCGAG CCGATCTGGG AGGAGCAGCA GTGCGAAGTG     480
AAGGCCGATG GCTTCCTCTG CGAGTTCCAC TTCCCAGCCA     520
CCTGCAGGCC ACTGGCTGTG GAGCCCGGCG CCGCGGCTGC     560
CGCCGTCTCG ATCACCTACG GCACCCCGTT CGCGGCCCGC     600
GGAGCGGACT TCCAGGCGCT GCCGGTGGGC AGCTCCGCCG     640
CGGTGGCTCC CCTCGGCTTA CAGCTAATGT GCACCGCGCC     680
GCCCGGAGCG GTCCAGGGGC ACTGGGCCAG GGAGGCGCCG     720
GGCGCTTGGG ACTGCAGCGT GGAGAACGGC GGCTGCGAGC     760
ACGCGTGCAA TGCGATCCCT GGGGCTCCCC GCTGCCAGTG     800
CCCAGCCGGC GCCGCCCTGC AGGCAGACGG GCGCTCCTGC     840
```

FIG. 9(b)

```
ACCGCATCCG CGACGCAGTC CTGCAACGAC CTCTGCGAGC     880
ACTTCTGCGT TCCCAACCCC GACCAGCCGG GCTCCTACTC     920
GTGCATGTGC GAGACCGGCT ACCGGCTGGC GGCCGACCAA     960
CACCGGTGCG AGGACGTGGA TGACTGCATA CTGGAGCCCA    1000
GTCCGTGTCC GCAGCGCTGT GTCAACACAC AGGGTGGCTT    1040
CGAGTGCCAC TGCTACCCTA ACTACGACCT GGTGGACGGC    1080
GAGTGTGTCG AGCCCGTGGA CCCGTGCTTC AGAGCCAACT    1120
GCGAGTACCA GTGCCAGCCC CTGAACCAAA CTAGCTACCT    1160
CTGCGTCTGC GCCGAGGGCT TCGCGCCCAT TCCCCACGAG    1200
CCGCACAGGT GCCAGATGTT TTGCAACCAG ACTGCCTGTC    1240
CAGCCGACTG CGACCCCAAC ACCCAGGCTA GCTGTGAGTG    1280
CCCTGAAGGC TACATCCTGG ACGACGGTTT CATCTGCACG    1320
GACATCGACG AGTGCGAAAA CGGCGGCTTC TGCTCCGGGG    1360
TGTGCCACAA CCTCCCCGGT ACCTTCGAGT GCATCTGCGG    1400
GCCCGACTCG GCCCTTGCCC GCTGA                   1425
```

FIG. 10(a)

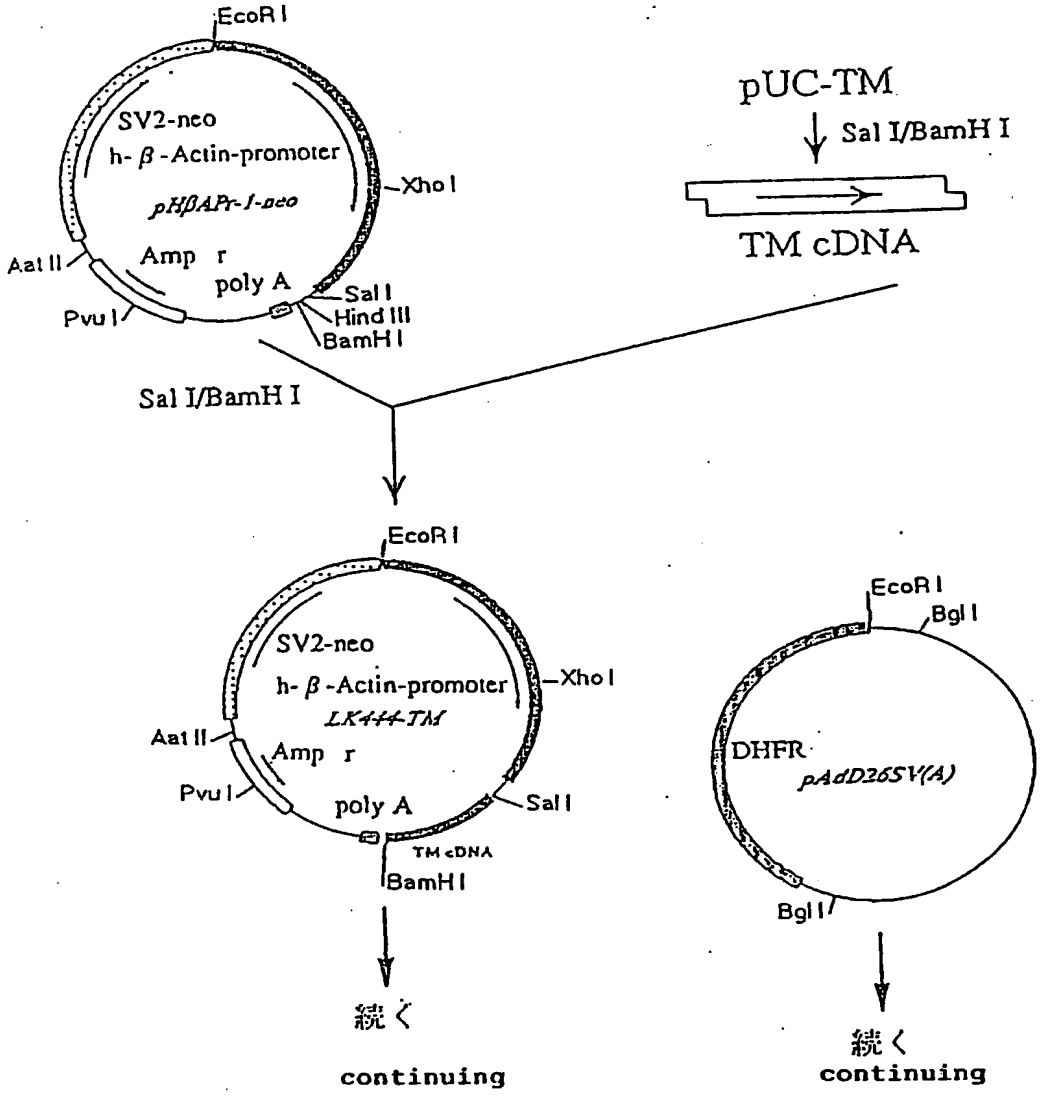

EcoR I
SV2-neo
h-β-Actin-promoter
pHβAPr-1-neo
Xho I
Aat II
Amp r
poly A
Sal I
Hind III
BamH I
Pvu I
pUC-TM
Sal I/BamH I
TM cDNA
Sal I/BamH I
EcoR I
SV2-neo
h-β-Actin-promoter
LK444-TM
Xho I
Aat II
Amp r
Pvu I
poly A
Sal I
TM cDNA
BamH I
続く
continuing
EcoR I
Bgl I
DHFR
pAdD26SV(A)
Bgl I
続く
continuing

FIG. 10(b)

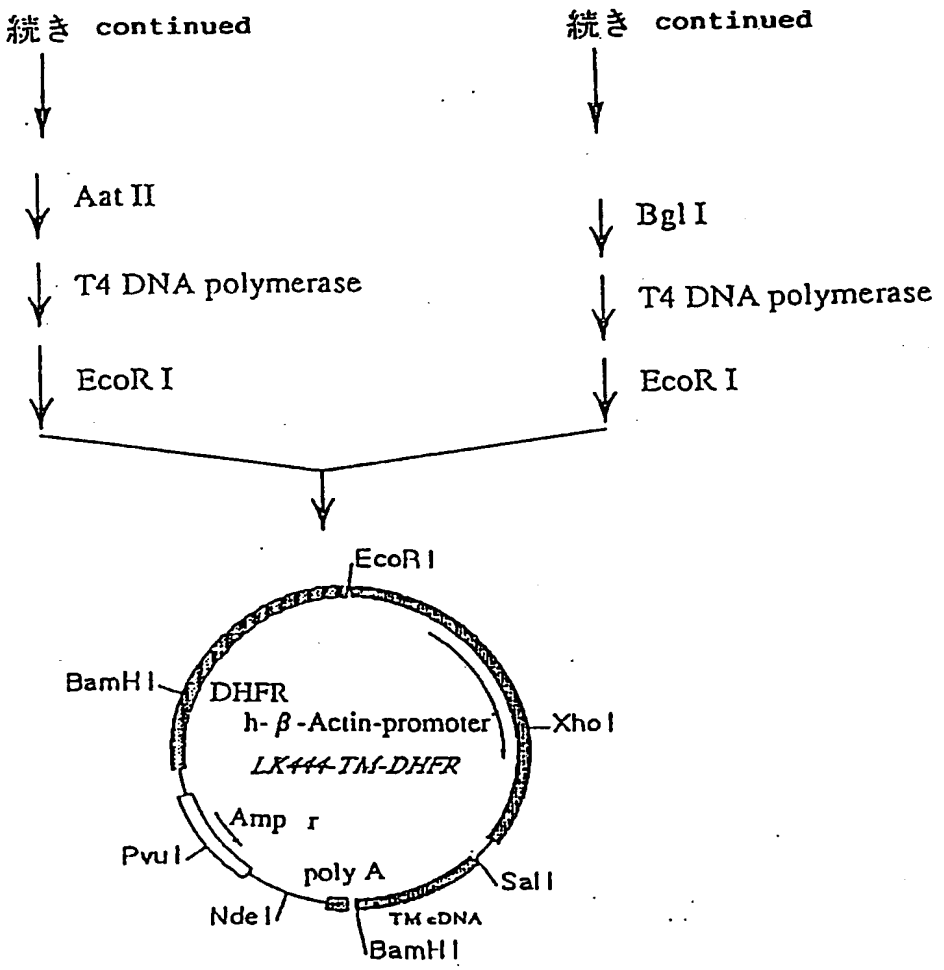

続き continued
続き continued
Aat II
T4 DNA polymerase
EcoR I
Bgl I
T4 DNA polymerase
EcoR I
EcoR I
BamH I
DHFR
h-β-Actin-promoter
LK444-TM-DHFR
Xho I
Amp r
Pvu I
poly A
Sal I
Nde I
TM cDNA
BamH I

FIG. 11(a)

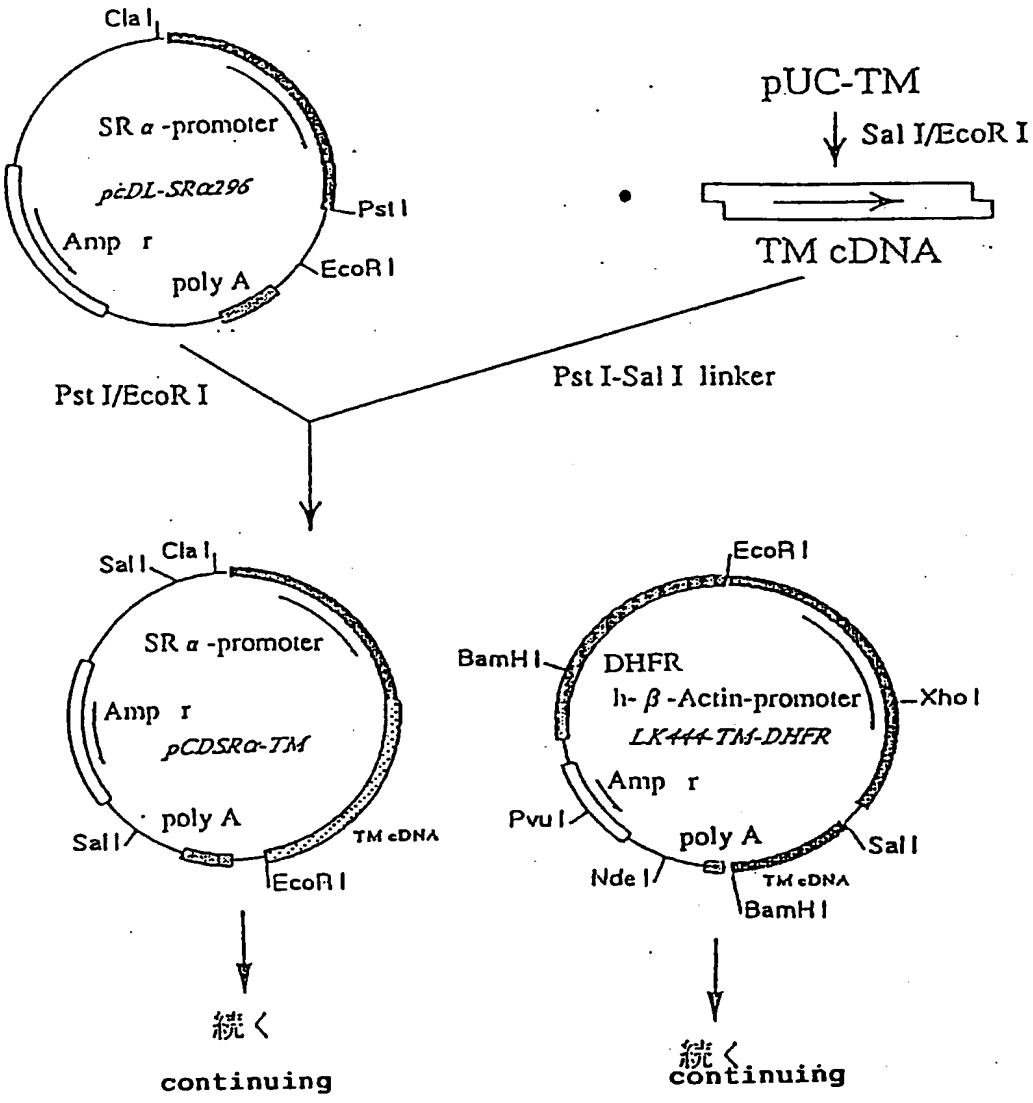

Cla I
SR α -promoter
pcDL-SRα296
Pst I
Amp r
EcoR I
poly A
pUC-TM
Sal I/EcoR I
TM cDNA
Pst I/EcoR I
Pst I-Sal I linker
Sal I
Cla I
SR α -promoter
Amp r
pCDSRα-TM
poly A
Sal I
TM cDNA
EcoR I
EcoR I
BamH I
DHFR
h- β -Actin-promoter
Xho I
LK444-TM-DHFR
Amp r
Pvu I
poly A
Sal I
Nde I
TM cDNA
BamH I
続く
continuing
続く
continuing

FIG. 11(b)

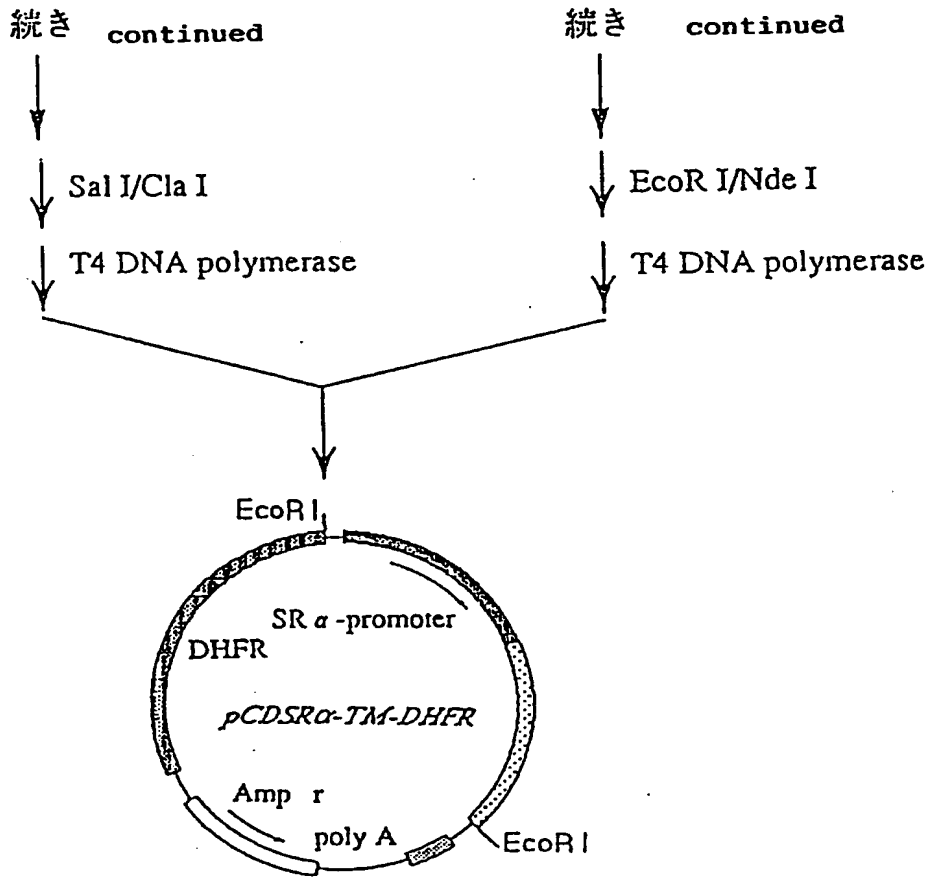

続き continued
Sal I/Cla I
T4 DNA polymerase
続き continued
EcoR I/Nde I
T4 DNA polymerase
EcoR I
SR α -promoter
DHFR
pCDSRα-TM-DHFR
Amp r
poly A
EcoR I

FIG. 12

（Ａ）ＤＥＬ１０作製用オリゴマー
oligonucleotides for DEL10 construction

D5-16U	CTTCGAGTGC TGATAG	16
D5-24L	AATTCTATCA GCACTCGAAG GTAC	24

（Ｂ）ＤＥＬ４９作製用オリゴマー
oligonucleotides for DEL49 construction

D10-14U	CTAGCTGTTG ATAG	14
D10-14L	AATTCTATCA ACAG	14

FIG. 13(a)

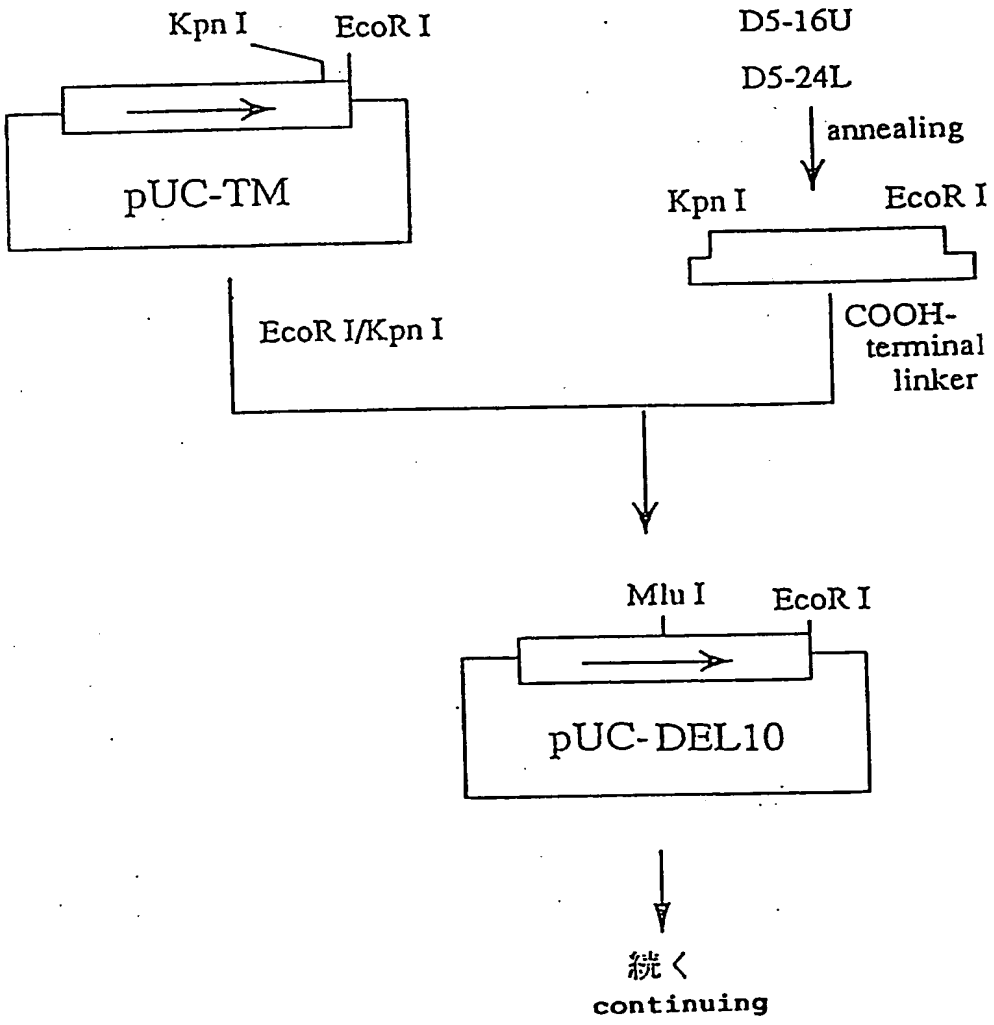
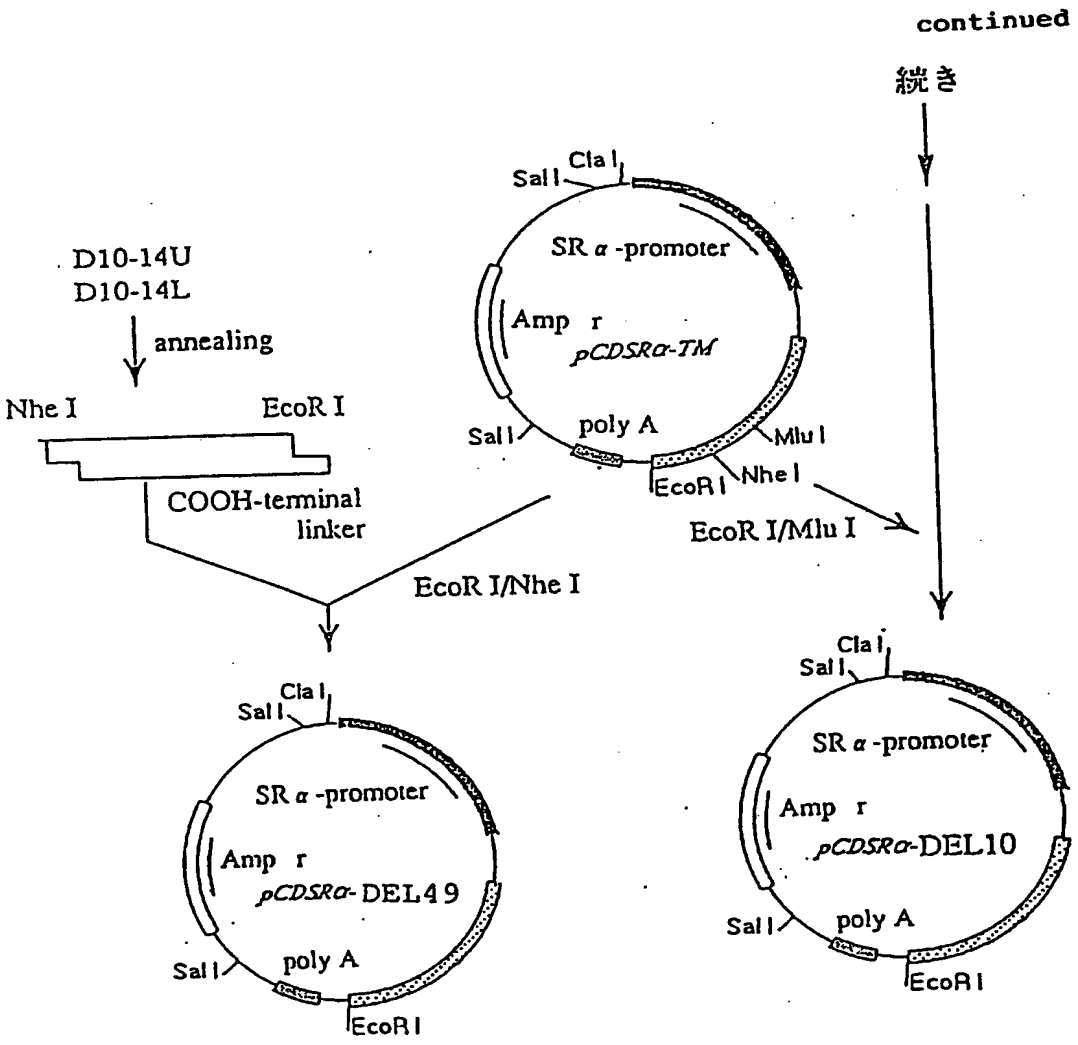

Kpn I
EcoR I
pUC-TM
D5-16U
D5-24L
annealing
Kpn I
EcoR I
EcoR I/Kpn I
COOH-terminal linker
Mlu I
EcoR I
pUC-DEL10
続く
continuing

FIG. 13(b)


continued
続き
D10-14U
D10-14L
annealing
Nhe I
EcoR I
COOH-terminal linker
EcoR I/Nhe I
Sal I
Cla I
SR α -promoter
Amp r
pCDSRα-TM
poly A
Mlu I
Nhe I
EcoR I
EcoR I/Mlu I
pCDSRα-DEL49
pCDSRα-DEL10

INTERNATIONAL SEARCH REPORT

International Application No PCT/JP91/00873

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC Int. Cl[5]

C07K13/00, 7/10, A61K37/02, C12N15/12, C12P21/02//(C12P21/02, C12R1:19) (C12P21/02, C12R1:91) C07K99:00

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC	C07K13/00, 15/06, 15/12, 15/14, C12N15/12, C12P21/00, 21/02

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

Biological Abstracts Data Base (BIOSIS)

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
P,X	EP, A2, 376251 (Mochida Pharmaceutical Co., Ltd.), July 4, 1990 (04. 07. 90), & CA, A, 2006658	1-4, 13-16 /5-12, 17
A	JP, A, 63-301791 (Washington University), December 8, 1988 (08. 12. 88), & EP, A2, 290419 & US, A, 4912207	1-17
A	Proceedings of the National Academy of Sciences, U.S.A., Vol.84, No.18, (1987), R.W.Jackman et al. "Human thrombomodulin gene is intron depleted:Nucleic acid sequences of the cDNA and gene predict protein structure and suggest sites of regulatory control" P.6425-6429	1-17
A	Biochemistry, Vol.26, No.14, (1987), D.Wen et al. "Human thrombomodulin complete complementary DNA sequence and chromosome localization of the gene" P.4350-4357	1-17

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
September 14, 1991 (14. 09. 91)	October 14, 1991 (14. 10. 91)
International Searching Authority	**Signature of Authorized Officer**
Japanese Patent Office	

Form PCT/ISA/210 (second sheet) (January 1985)

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FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

A	EMBO Journal, Vol.6, No.7, (1987), K.Suzuki et al. "Structure and expression of human thrombomodulin A thrombin receptor on endothelium acting as a cofactor for protein C activation" P.1891-1898	1-17

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers . because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claim numbers , because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claim numbers , because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

1 ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application

2 ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3 ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest

☐ No protest accompanied the payment of additional search fees.